UNIVERSITY OF ALBERTA

Nitric Oxide and Matrix Metalloproteinases as Mediators of Chronic and Acute Inflammatory Disorders in Humans:

Focus on Chronic Liver Damage and Acute Lung Injury

by

Lance McNaughton

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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ABSTRACT

Inflammation is a fundamental biological process aimed at protecting the host from various types of injury. Normally an ameliorative process, inflammation may in some cases become uncontrolled, self-perpetuating and destructive, leading to tissue and organ damage. Though there are as many as 30 distinctly recognized forms of inflammation, variations in the duration and extent of tissue damage account for only a few clinically defined inflammatory states, i.e. acute, subacute or chronic inflammation.

The mechanisms of inflammatory reactions include dynamic interactions between various participating systems and agents. This thesis deals with the characterization of crucial inflammatory mediators such as interleukin-6 (IL-6), nitric oxide (NO) and matrix metalloproteinases (MMP-2 and MMP-9) in chronic and acute inflammatory reactions in humans. The research has focused on chronic end-stage liver disorders and acute lung injury as clinical examples of chronic and acute inflammation.

Normal human liver expressed both endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). The expression of iNOS was strong in the periportal regions and weak in the perivenous regions of liver acini. Chronic end-stage liver disorders including viral hepatitis, alcoholic cirrhosis, cholestasis, α_1-antitrypsin deficiency and haemochromatosis were found to be associated with increased expression of iNOS and nuclear translocation of eNOS. Increased expression of iNOS was not associated with changes in the activity of arginase, an enzyme that competes with NOS for L-arginine substrate. In addition to NOS, end stage liver disorders were associated with increased expression of MMP-2.

Acute lung injury brought about by primary lung disorders as well as by disease states in which the lungs were secondarily involved led to an increased release of IL-6, NO, MMP-2 and MMP-9 in plasma. No significant correlations were detected between the levels of these mediators and the indices of patient well being such as the APACHE II score.

It is concluded that, similar to animal models of inflammation, chronic and acute inflammatory reactions in humans lead to activation of both the NO and MMP-dependent pathways of inflammation. The pathogenetic and therapeutic significance of these findings remain to be studied.

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Nitric Oxide and Matrix Metalloproteinases as Mediators of Chronic and Acute Inflammatory Disorders in Humans: Focus on Chronic Liver Damage and Acute Lung Injury* by Lance McNaughton, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

My last 5 years at the University of Alberta have left me with many pleasant memories and a world of new friends. As a graduate student, I quickly recognized that my accomplishments in the program would ultimately be defined by my own personal limitations. Rising to many challenges, my long hours and erratic schedule often meant sacrificing priceless family time. While I will never get that back, I thank God that I have been blessed with a loving and supportive family who has always been there for me, each and every single step of the way.

I am also privileged to say that my graduate studies experience has changed my life in a way I never could have imagined. It was here that I met, and later married my charming wife María Angeles, and more recently we were blessed with the birth of our darling daughter, Ariana. Thanks to the two of you, the understanding, patience and support you have given me throughout the remainder of my Ph.D. studies has instilled in me a newfound strength, happiness, and love for life!

Forever grateful, I dedicate this work to all of you, my family.

PREFACE

This thesis focuses on inflammatory diseases in humans. In the first chapter a brief overview of the inflammatory process is provided. The two major divisions of my investigation appear in chapters II and III and consist of *chronic and acute inflammatory liver disease* and *acute inflammatory lung disease*, respectively. Each of these two chapters is presented with *specific background information* followed by *research objectives*, *materials and methods*, *results* and *discussion* sections. The final chapter consists of a *bibliography* for all literature that is cited throughout the thesis.

ACKNOWLEDGMENTS

This study would not have been possible were it not for the dedication, spirit and vision that was shared by each of my committee members and friends: Dr. Marek Radomski, Dr. Bozena Vollrath, Dr. Irvin Mayers, Dr. Norman Kneteman and Dr. Richard Schulz. Your support has been a true inspiration and has been integral to ensuring the overall development, coordination, integrity and success of my Ph.D. research project. Also, I wish to extend a very special thanks to my supervisor Dr. Marek Radomski and co-supervisor Dr. Bozena Vollrath, whose endless patience, friendship and guidance will never be forgotten.

During the course of my graduate studies I have learned a great deal from many of the people that have worked in our department and lab. While there are too many to mention here, I wish them all to know how grateful I am to have made their acquaintance and shared many insightful conversations. Of course a million thanks go out to my dear friends who currently work in our lab: Dr. Marek Radomski, Dr. Anna Radomski, Dr. Grzegorz Sawicki and Paul Jurasz.

As the setting for this study, the University of Alberta and the University of Alberta Hospital (UAH) provided state of the art facilities and knowledgeable staff that together are recognized for the highest standards in health science. It was truly a privilege to be associated with the entire organization and I would like now to name some of the UAH departments and staff whose behind-the-scenes efforts since this work began in November 1996 deserve full recognition.

I am deeply grateful to Dr. Norman Kneteman (Surgical Director of Transplantation at the UAH) who, along with his skilled team of surgical associates, provided some of the crucial tissue samples that were a major component of this study. Likewise, the help and guidance of Dr. Irvin Mayers in executing the ICU study was most appreciated. The logistics of obtaining human liver and blood specimens required the coordinated efforts of many individuals and these gentlemen provided a crucial link in the chain that always delivered.

Thanks also to Ingrid Larsen (former UAH Recipient Coordinator) and her group from the University of Alberta Hospital Liver Transplant Program whose assistance in patient recruitment and specimen collection was indispensable. In addition I wish to thank the countless nursing staff on UAH patient care units 4A1 and 3E3 whose unlimited assistance made the collection of patient blood samples a well organized success.

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Happiness and health to you all,

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LIST OF ACRONYMS, NOMENCLATURE OR ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AC	Alcoholic cirrhosis
ACTH	Adrenocorticotropic hormone
AI-Hep	Autoimmune hepatitis
ALT	Alanine aminotransferase
AP	Alkaline phosphatase
APACHE	Acute physiology and chronic health evaluation
ARDS	Adult respiratory distress syndrome
ASA	Acetylsalicylic acid
AST	Aspartate aminotransferase
CC	Cryptogenic cirrhosis
CD	Cluster of differentiation
cDNA	Complementary DNA
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disorder
COX	Cyclooxygenase
Cre	Creatinine
CsA	Cyclosporine-A
CSF	Colony-stimulating factor
Cys	Cysteine
DNA	Deoxyribonucleic acid
ECMO	Extracorporeal membrane oxygenation
EDRF	Endothelial-derived relaxing factor
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FHF	Fulminant hepatic failure
FMN	Flavin mononucleotide
HEHE	Hepatic epithelioid hemangioendothelioma

HPLC	High-performance liquid chromatography
GI	Gastrointestinal
H_2O_2	Hydrogen peroxide
HC	Haemochromatosis
ICAM	Intercellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
kDa	Kilodalton
K_i	Inhibitor constant
K_M	Michaelis constant
LFA	Lymphocyte function-associated antigen
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NOHA	N^{G}-hydroxy-L-arginine
LPS	Lipopolysaccharide
LT	Leukotriene
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-MMP	Membrane-type MMP
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic non-cholinergic
NFκB	Nuclear factor kappa-B
NMDA	N-methyl D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO-NSAIDs	Nitric oxide coupled NSAIDs
NOS	Nitric oxide synthase
NO_x^-	Nitrate and nitrite
NSAIDs	Non-steroidal anti-inflammatory drugs

O_2^-	Superoxide
OH·	Hydroxyl radical
OLT	Orthotopic liver transplant
$ONOO^-$	Peroxynitrite
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBC	Primary biliary cirrhosis
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PL	Phospholipase
PECAM	Platelet-endothelial cell adhesion molecule
proMMP	Proenzyme isoform of matrix metalloproteinase
PSC	Primary sclerosing cholangitis
RES	Reticuloendothelial system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SOD	Superoxide dismutase
TGF	Transforming growth factor
Th	T-helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
TX	Thromboxane
VH	Viral hepatitis

Chapter I

GENERAL INTRODUCTION

INFLAMMATION

First century AD Greek and Roman physicians were among the first to describe inflammation, a process they perceived as a single disease entity. Historically, the term was itself derived from one its symptoms: heat; L. *inflammo*, pp. *-atus*, fr. *in*, in, + *flamma*, flame. In all a total of five symptoms were identified as shown in Illustration I-1 below: *calor/heat, rubor/redness, dolor/pain, tumor/swelling and functio laesa/loss of function*, in Greek and English, respectively. It is interesting to note that it was not until the dawn of modern pathology in 1858 that the latter symptom was added to the original four as seen in Virchow's Cellular Pathology.[1]

Illustration I-1

Remarkably, the symptoms of inflammation as described by classic physicians are still used today to characterize this pathologic process irrespective of the factor(s) that trigger the inflammatory reactions. Indeed, the stimuli that initiate the inflammatory reactions are diverse: endogenous factors may include immunopathological reactions as well as some genetic and neurological disorders whereas exogenous factors include those of mechanical (e.g. trauma), physical (e.g. radiation or temperature), chemical (e.g. toxins), nutritive (e.g. oxygen, vitamins) or biological (e.g. bacterial or parasitic) basis. Factors relating to the severity of inflammation depend on the causative agent, the location of the injury and the composition of the inflammatory exudate. Perhaps most important is the duration of the inflammatory process, which, for scholarly purposes, is often categorized as either *acute, subacute* or *chronic.*[2] When natural healing or early anti-inflammatory therapy fails, the acute stage, characterized by neutrophil infiltration and oedema, gradually gives way to a more aggressive immune response. Subacute inflammation refers to a mild, protracted state of acute [L. *acutus,* sharp] inflammation. In chronic [G. *chronos,* time] inflammation, tissue destruction and inflammation proceed at the same time as tissue healing. This leads to excessive proliferation of connective tissue cells resulting in a notable increase in extracellular matrix proteins (e.g. collagens, glycoproteins and fibronectin). As well, a mononuclear cell infiltrate consisting of macrophages and lymphocytes is a definitive shift in the immune cell phenotype that is characteristic of chronic inflammation. In some specific diseases such as tuberculosis, leprosy and schistosomiasis, the accumulation of modified macrophages called epithelioid cells at the site of tissue injury results in a persistent nodular inflammatory lesion known as a granuloma.[3] Resolution of a chronically inflamed lesion does not always guarantee a complete recovery- the ingrowth of fibrous tissue may often result in residual scarring.

Just as the inflammatory process can be subdivided into acute, subacute and chronic states, the actual initiation of the inflammatory response can be broken down into separate stages. First, the body responds to chemical substances released by damaged tissue cells, through neural and hormonal mechanisms, during which time the lymphoreticular system amplifies its phagocytic and antibody generating cell regiment. At the time of injury, blanching or whitening of the skin is indicative of an early transient state of vasoconstriction that serves to minimize the initial extent of hemorrhage. Within seconds, however, this short-lived response is quickly replaced by the acute vascular response in which activation of the endothelial cells bordering the lesion promotes vasodilatation (hyperaemia and erythema) and increased capillary permeability (oedema). Consequently, this altered haemodynamic state facilitates the hallmark accumulation and infiltration of granulocytes, particularly neutrophils, and persists as long as tissue damage is evident. (for review, see Guyton AC)[4]

In general, inflammation may be described as a complex response launched by the body following damage to cells and vascularized tissues. As such, true inflammation is not possible in avascular tissues, e.g. in normal cornea. The survival advantage of the inflammatory process is to contain, neutralize or limit the effects of injurious factors and ultimately restore homeostasis to the affected tissue(s). At the same time, the closely-knit actions of the immune system commission many of the same molecules to prevent invasion of the weakened tissue by foreign organisms. Remarkably, that such actions are both ontogenetic and phylogenetic supports the role of inflammation as a one of the oldest and most highly essential biological responses.

In light of the immeasurable benefits of the inflammatory reaction to injury, it may at first seem rather ironic that a large branch of medical science is devoted to the ongoing

development of increasingly potent anti-inflammatory therapies. The logic behind such efforts lies in treating a pathologic extension of inflammation in which the inflammatory process itself becomes self-perpetuating rather than self-limiting, and this leads to permanent cell and tissue injury. Clinically, such injuries underlie the development of the fifth symptom of inflammation: loss of function. Rapidly developing reactions such as endothelial injury, leaky vasculature and increased inflammatory cell infiltration all may lead to a state of microcirculatory collapse similar to what occurs in sepsis, shock, asthma, ischemia and graft rejection. The most extreme example of endothelial dysfunction is seen when the endothelial lining undergoes global activation as seen in conditions such as systemic inflammatory response syndrome[5] and disseminated intravascular coagulation.[6] Indeed, inflammation in all its stages has various clinical forms: e.g. a chronic debilitating disease like arthritis or gout or an acute life-threatening condition such as anaphylactic shock. These pathologies exemplify the serious consequences of the body's loss of control over its self-defense reactions. While the reasons for this phenomenon are still unclear, studies over the last thirty years have been of paramount importance since they have characterized some of the mechanisms involved in the underlying cascade of inflammatory reactions. Nevertheless, despite decades of research it is important to recognize that current anti-inflammatory therapy still relies primarily on the use of non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids that treat the symptoms but not the cause of inflammation. Successful causative treatment of the underlying disorder depends not only on an accurate diagnosis but also on the availability, efficacy and compatibility of appropriate drugs.

To better understand inflammation it is helpful to classify the participating molecules by their predominant action(s), though such simplification is not intended to suggest their exclusivity to

a single category. In fact, the cross-talk between the molecules involved in inflammation is so common that it is not possible to offer a clear account of any one molecule's actions. Overall, three major groups will be highlighted: ***biochemical regulators***, ***mediators*** and ***inhibitors***. The following discussion will focus on some of the key actions of these molecules relevant to inflammation in order to illustrate the myriad of biochemical pathways that currently represent viable targets of anti-inflammatory agents.

Biochemical Regulators

Under physiologic conditions, the mosaic of anti-inflammatory processes that accompany tissue injury should gradually decay to a point of extinction by the time that regeneration of healthy tissue(s) has occurred. When this regulatory process fails to happen and the responsible mediators continue to act without due control, the acute inflammatory process becomes protracted resulting in short term (sub-acute) or long term (chronic) variations of inflammation. Fortunately, the body employs a number of biochemical-based systems with which it quite successfully regulates the actions of inflammatory mediators throughout all stages of the inflammatory process. Interestingly, compared with the large number of inflammatory mediators and inhibitors, there are only two major regulatory systems of the inflammatory response: adrenal hormones such as glucocorticoids and a sizeable group of signalling peptides known as cytokines.

Glucocorticoids

Injury or infection evoke an inflammatory response which is immediately modulated by glucocorticoids released from the adrenal cortex. In addition to their direct anti-inflammatory effects, glucocorticoids also affect carbohydrate metabolism, bone resorption, protein

catabolism and fat redistribution. For these reasons, the pharmacological use of glucocorticoids like hydrocortisone as anti-inflammatory agents is limited by the appearance of many unwanted side effects. When used over a prolonged period, pharmacological doses of glucocorticoids may lead to Cushing's syndrome.

Glucocorticoids are cholesterol-based molecules that readily cross cell membranes where they activate *intracellular* receptors. Once bound, the activated receptor-hormone complex translocates to the cell nucleus resulting in activation or inhibition of specific gene transcription processes. The anti-inflammatory actions of corticosteroids are achieved through inhibition of cytokine synthesis, inhibition of neutrophil and monocyte activation, as well as decreased T and B cell responsiveness. In addition, glucocorticoids induce the synthesis of a specific protein, lipocortin, that inhibits the activity of phospholipase A_2 (PLA_2) and the downstream production of eicosanoids and platelet activation factor controlled by PLA_2. Lipocortin exerts a number of anti-inflammatory effects and, in contrast to glucocorticoids, is largely deprived of metabolic actions. Despite this attractive pharmacologic profile, the use of lipocortin as an anti-inflammatory agent that lacks the side effects of glucocorticoids has fallen short of glucocorticoid potential. Indeed, this can best be explained by the multiple anti-inflammatory actions of corticosteroids that often represent a powerful last resort to anti-inflammatory therapy. (for review, see Peers SH et al.)[7]

Cytokines

No discussion about inflammation is complete without referring to cytokines. Despite their many overlapping functions, cytokines may be arbitrarily grouped into the following four broad categories of function: (1) regulators of immune-mediated inflammation, (2) regulators of cell-mediated or humoral immunity, (3) mediators of natural immunity, and (4) stimulators

of immature leukocyte growth and differentiation. Cytokines are low molecular weight peptides (less than 80 kDa) that are transiently secreted by many cell types following *de novo* synthesis. Once generated, they act via autocrine, paracrine or endocrine pathways to activate high affinity receptors and liberate second messengers, thus affecting nuclear genetic expression. At remarkably low concentrations cytokines can influence the function of virtually any cell type, and this ability underlies their pivotal role in many cellular pathologies. These properties of cytokines account for the immense pharmacologic interest in seeking ways to attenuate their proinflammatory actions.

Over the last fifty years the study of infectious disease and antigen-induced immune responses has contributed greatly to the characterization of cytokines. Perhaps the greatest advancements came in the 1980's with the development of *cytokine cloning and expression systems*. Since then it has become increasingly evident that cytokines are truly multi-faceted molecules by virtue of their diverse cellular sources, redundant and pleiotropic character, ability to produce multiple different effects on the same target cell and to influence the synthesis and actions of one-another. Fortunately, the complex array of cytokine activities has become somewhat more intelligible by grouping them on the basis of their patterns of release. One classic model of immune system function proposes the existence of two distinct cytokine-based responses to inflammatory stimuli. This model accounts for the first two categories of cytokines described above: (1) regulators of immune-mediated inflammation and (2) regulators of cell-mediated or humoral immunity. The model is based on the specific T-helper cell immune responses giving rise to Th1 and Th2 cells and the cytokine expression patterns associated with these cells. The process of T-helper cell differentiation begins with naive $CD4^+$ T-cells which, upon exposure to a foreign antigen, produce interleukin-2 (IL-2) and

undergo a conversion to a Th0 phenotype which secretes a discrete variety of cytokines. Depending on the nature of both the antigen and the cytokine releasate, the Th0 cells then differentiate into either a Th1 cell population or a Th2 population. Th1 cells respond to and release a specific pool of cytokines including IL-2, IL-12, and interferon-γ (IFN-γ). As such they are the principal effectors of cell-mediated immunity against intracellular microbes and autoimmunity, both involving macrophage activation and free radical evolution. The cytokines they release lead to the production of antibodies capable of activating complement and opsonizing antigens for phagocytosis. In general, the Th1 response retards the healing process and is proinflammatory in nature.

Th2 cells respond to and release a specific pool of cytokines including IL-4, IL-5, IL-8, IL-10, IL-13 and transforming growth factor-β (TGF-β). In several instances they produce the opposite effects of their Th1 counterparts, including macrophage inactivation, promotion of healing and in some cases over-healing, scarring or infection. In particular, Th2 cytokines are associated with humoral immunity (phagocyte-independent) and play a particularly important role in helminthic disease and allergic and atopic reactions by promoting IgE release and eosinophil activation. Illustration I-2 highlights the different pathways associated with the Th1/Th2 inflammatory response.

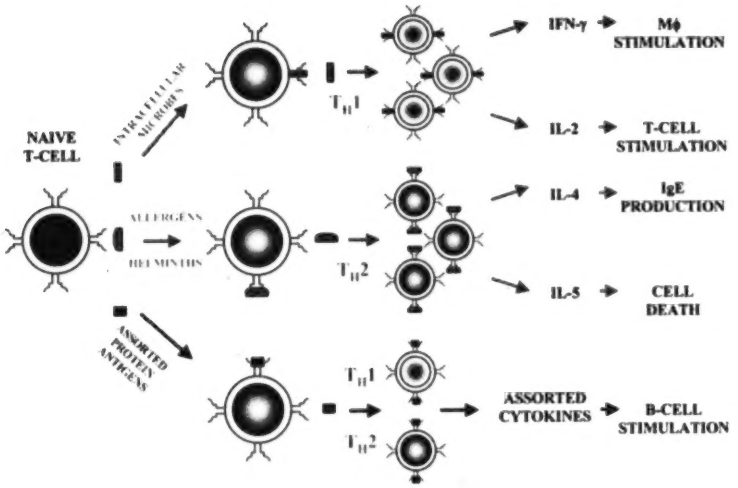


Illustration I-2: **T_H1/T_H2 INFLAMMATORY PATHWAYS** Different antigenic stimuli determine a specific pathway of differentiation and effector functions of a naïve CD4+ T cell. Cells infected with intracellular microbes stimulate the naïve T cell (upper pathway) to differentiate into a poplulation of T_H1 clones that produce a phagocyte mediated response through the actions of interferon-γ (IFN-γ) and interleukin-2 (IL-2) on macrophages (MΦ) and other T cell lineages. Parasites and allergens induce the middle pathway- T_H2 clones proliferate and produce a phagocyte-independent response via the effects of interleukins-4 (IL-4) and -5, (IL-5) that result in IgE production and eosinophil stimulation. The lower pathway inyolves assorted protein antigens and leads to the generation of both T_H1 and T_H2 lineages resulting in a humoral immune response.

The cytokines belonging to the third group are well recognized for their role as early phase mediators of the acute inflammatory response and natural immunity. In particular, these cytokines include the type I interferons (α and β subtypes) which are primarily associated with viral infections, and the proinflammatory cytokines tumor necrosis factor-α (TNFα), IL-1 and IL-6. Given the relative importance of this group of cytokines in the acute inflammatory response, the remaining discussion will focus on some of the most important actions of TNFα, IL-1 and IL-6.

An early event in the inflammatory response is the rapid release of TNFα from mononuclear phagocytes arriving at the site of injury. The release interval and resultant peak concentration of TNFα significantly influences natural and acquired immunity and acute inflammation processes. Development of a fever is a common symptom of injury which results from

TNFα-mediated induction of cyclooxygenase-2 and generation of prostaglandins, particularly prostaglandin E_2 (PGE_2). The studies on the Schwartzman reaction, often compared to an exaggerated host response to infection that is characterized by a burst in TNFα levels, have revealed the multifactorial actions of TNFα. This cytokine causes activation of coagulation pathways, endothelial cells, leukocytes, monocytes and lymphocytes that are accompanied by a temporal wave of IL-1 and IL-6 release. The release of these cytokines may serve to down-regulate the effects of TNFα. Indeed, the rise in plasma IL-1 and IL-6 causes the adrenal pituitary axis to discharge adrenocorticotropic hormone (ACTH) that in turn activates the adrenal cortex to release hydrocortisone. This glucocorticoid then acts on TNFα-producing cells to decrease the generation of this cytokine. Like many cytokines, the multiple actions of TNF make it a difficult target for pharmacologic modulation given that inadequate TNF levels are characterized by a failure to contain infections while high amounts lead to cell damage and death.

Adding to the effects of TNF, IL-1 functions in natural immunity as a mediator of the host inflammatory response. It has been suggested in fact that these two cytokines may play a central role in chronic inflammatory processes such as those seen in rheumatoid arthritis.[8] In sufficiently high concentrations, IL-1 becomes endocrine-like, and much like TNF, promotes acute phase protein synthesis and pyrogenesis. Interestingly, IL-1 is the one of few cytokines for which a naturally occurring inhibitor has been discovered.[9]

The last early phase mediator to be released is IL-6. Among many, two of the best-described actions of IL-6 are stimulation of hepatocyte-mediated acute phase protein synthesis[10] and B cell differentiation. An overview of the early cascade of proinflammatory cytokines released during sepsis is depicted in Illustration I-3. From a clinical point of view it would appear that

plasma levels of each cytokine might provide clues to the stage and severity of an acute injury. This theory is partially supported by the observation that high concentrations of TNF underlie the development of septic shock syndrome.[11]

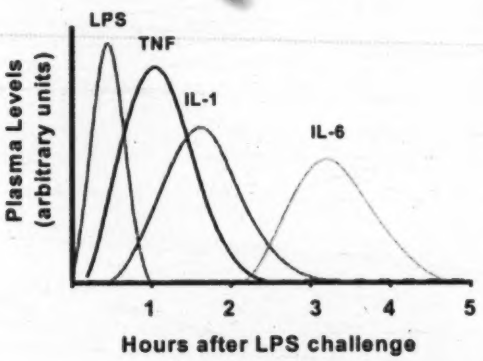


Illustration I-3: **CYTOKINE CASCADES IN SEPSIS** Illustration shows plasma levels of tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) during the first 5 hours following the injection of lipopolysaccharide (LPS) into a rat. In the absence of LPS, injection of TNF produces the same successive downstream cascade of IL-1 and IL-6.

The fourth class of cytokines consists of mediators of immature leukocyte activation, growth and differentiation, most of which are collectively known as colony-stimulating factors (CSFs). In general, production of these cytokines occurs during natural and antigen-induced immune responses and serves to stimulate hematopoiesis of bone marrow progenitor cells. Perhaps the most versatile of these is IL-3, a multilineage CSF that is mainly produced by $CD4^+$ T-cells and promotes the generation of virtually all known mature cell types. The actions of other CSFs including granulocyte-macrophage colony-stimulating factor and IL-7 act on progenitor cells at different stages and thereby selectively promote development of colonies of different lineages. (for review, see Abbas AK et al.)[12]

In addition to their many direct pro-inflammatory effects, cytokines also trigger the release of many mediators of inflammation. Several major classes of proinflammatory mediators and

inhibitors are now recognized, including the acute phase proteins, adhesion proteins, neuropeptides, biogenic amines, complement proteins, kallikrein-kinin peptides, blood coagulation factors, eicosanoids, platelet-activating factor, reactive oxygen species and cellular proteases. Each of these classes will now be briefly described in order to emphasize their role as *effectors* of inflammatory processes as opposed to the *regulators* mentioned above.

Biochemical Mediators and Inhibitors

Duplication of function, a salient feature of this large class of molecules, underscores the importance of the inflammatory response to the survival of the organism. Generated either locally or systemically, the mediators and inhibitors of the inflammatory response act through a comparably small number of cell types. In particular, only four major cell types account for the physical changes of inflammation. These include *vasodilatation* mediated via vascular smooth muscle cells, increased *vascular permeability* via cytoskeletal contraction in endothelial cells, *chemotaxis* of phagocytic leukocytes, and *phagocytosis* by activated macrophages.[13] As the following section will show, these substances may be generated in a variety of ways: local activation of circulating zymogens; release of preformed molecules from intracellular granules; or *de novo* synthesis. A brief review of the individual actions of each inflammatory mediator or inhibitor in the context of current pharmacotherapeutic paradigms will provide a background and rationale for this thesis investigation.

Acute Phase Proteins

The acute phase proteins are a diverse group of approximately thirty plasma proteins produced by the liver in response to inflammatory cytokines (TNFα, IL-1β and IL-6) that are liberated during the acute inflammatory response. Acting as either mediators, inhibitors or scavengers

of the acute inflammatory process, these proteins reach peak plasma levels anywhere from 6-72 hours following tissue injury. Regardless of the nature of the lesion, the plasma levels of C-reactive protein and serum amyloid A protein are most dramatically elevated (up to 1000-fold), followed by haptoglobin, complement components, and fibrinogen (2-4-fold). The major effect of activated C-reactive protein (antigen bound) is to stimulate the classic pathway of complement activation via the C1q glycoprotein. Serum amyloid-A and haptoglobin act as scavengers of the inflammatory by-products cholesterol and haemoglobin, respectively. Removal of these substances aids in tissue healing since they accumulate throughout the course of the inflammatory process: phagocytosis of cell debris leaves behind cholesterol and microvascular damage and coagulation liberates haemoglobin. Fibrinogen aids in the repair process by actively participating in blood clot formation. Among the multitude of other acute phase proteins it is worth mentioning that many serve as either specific or non-specific inhibitors of protease activity, and therefore provide systemic protection from these enzymes. Interestingly, a diminished acute phase protein response has been associated with various chronic inflammatory disorders like systemic lupus erythematosus, ulcerative colitis, primary biliary cirrhosis and hepatitis.[14] (for review, see Abbas AK et al.)[12]

Adhesion Proteins

Cell migration and recognition is essential to many biologic processes such as embryogenesis, tissue repair and immune and inflammatory responses. In particular, the adhesion of leukocytes to the postcapillary venule of the microvasculature is a characteristic part of the acute inflammatory response. In contrast, lymphocytes rarely adhere to inflamed endothelium.[15] The overall process may be simplified into 3 stages: tethering and rolling, activation, and firm adhesion allowing transmigration (Illustration I-4).

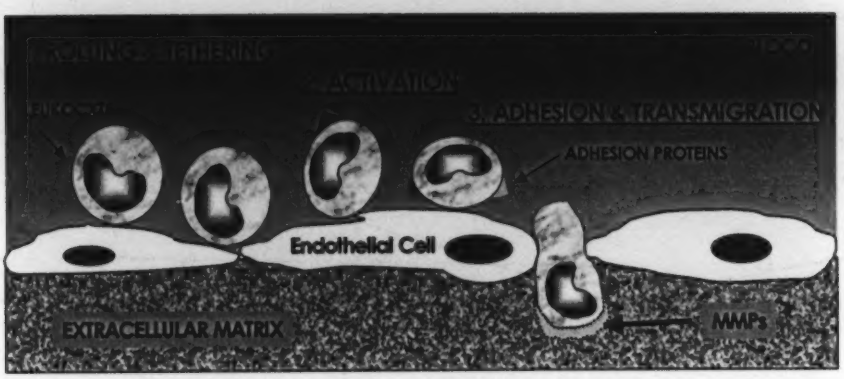


Figure I-4: **EARLY STAGES OF LEUKOCYTE MIGRATION** Three major stages of leukocyte migration into the extravascular space are shown. Early rolling and tethering interactions between leukocytes and activated endothelial cells lead to their activation and subsequent adhesion and transmigration into the extracellular matrix. Adhesion proteins and matrix metalloproteinases (MMPs) play critical roles in these processes.

A number of important factors known to influence various stages of the process include expression of adhesion molecules on the interacting cells, cytokine and chemokine environment, products of leukocyte (e.g. superoxide) and endothelial cell (e.g. nitric oxide) activation, and local fluid dynamics. The last stage of the process involves extravasation of the leukocyte through the endothelial cell layer and adjacent basement membrane into perivascular tissue. Such actions are achieved through signaling pathways involving cell-cell interactions and matrix metalloproteinase (MMP) secretion by the invading cell. In general, the importance of adhesion molecules is reflected by their enhancement of receptor-ligand interactions and mediation of important effector functions via intracellular signaling pathways.

Four adhesion molecule superfamilies have been well characterized and these include the selectins, the integrins, certain immunoglobulins and the cadherins. Because activated leukocytes and endothelial cells are capable of expressing various types of adhesion proteins on their outer surface, their morphology combined with the nature of the stimulus will largely influence the variety of molecules produced and the cell-cell interactions that follow the expression of adhesion molecules on the surface of interacting cells (Illustration I-5).

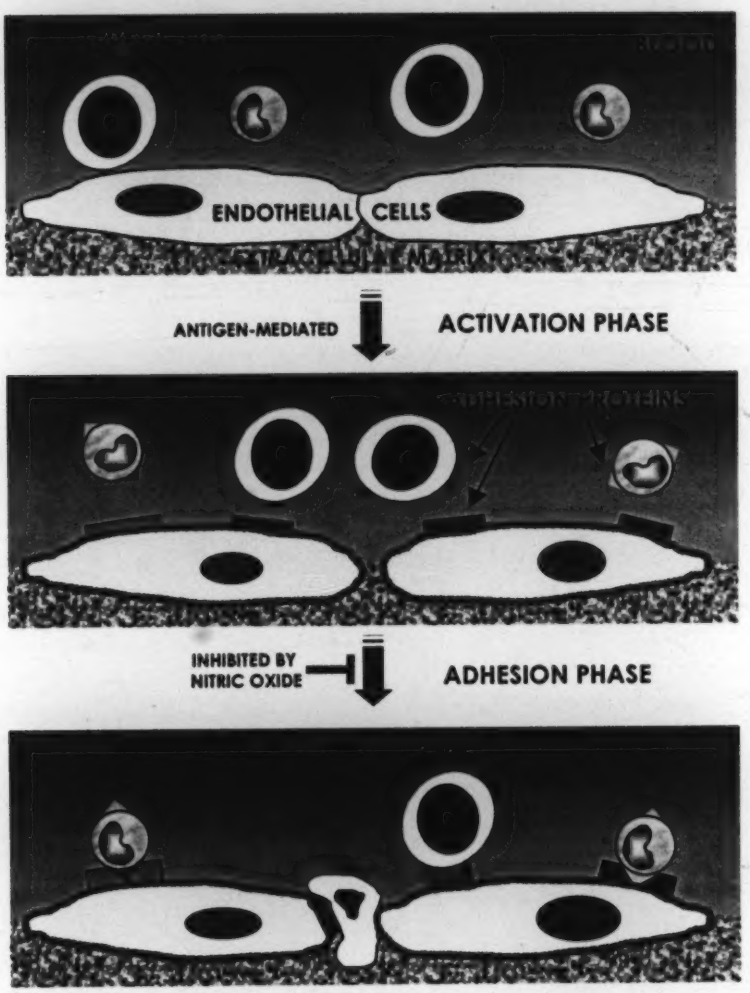


Illustration I-5: **RECEPTOR-LIGAND INTERACTIONS OF THE ENDOTHELIUM AND LEUKOCYTES**
Top image shows various leukocytes in a blood vessel along with local endothelial cells and the adjacent extracellular matrix. Following exposure to an antigen (upper arrow), these cells become activated and express a variety of adhesion proteins (center image). This activation promotes leukocyte-endothelial cell interactions via complementary adhesion proteins (lower image). This process of leukocyte adhesion is inhibited by nitric oxide (lower arrow).

The selectins consist of three closely related adhesion proteins whose names are derived from the cells in which they were first discovered. Constitutively expressed on leukocytes, L-selectin (CD62L) targets activated endothelial cells and facilitates leukocyte rolling. In a reciprocal interaction, E-selectin (CD62E) is produced exclusively by cytokine-activated endothelial cells and helps the endothelial cells to tether leukocytes. The third selectin also involved in

leukocyte binding is P-selectin (CD62P). In platelets, P-selectin is preformed and stored for rapid release in the α-granules. In the endothelium, P-selectin is stored in Weibel-Palade bodies and its induction or release follows cytokine-mediated activation.

The integrins consist of a large group of heterodimeric glycoproteins (gp) composed of alpha (α) and beta (β) class monomers that firmly adhere leukocytes and lymphocytes to the endothelium or various cell matrix proteins including collagen, laminin, fibronectin etc. Platelet function also depends on integrins; the gpIX/Ib (CD 42AB) complex promotes von Willebrand factor binding and adhesion to the subendothelium while the gpIIb/IIIa (CD41/61) complex is an important mediator of platelet aggregation. Exposure to β_1 integrins stimulates T cells to secrete MMP-2 and MMP-9, two important metalloproteinases with a special ability to degrade the basement membrane.

Transmigration of leukocytes across the endothelium is largely the result of interactions between the leukocyte integrin LFA-1 and intercellular adhesion molecules like ICAM-1 (CD54) or ICAM-2 (CD102), both a part of the immunoglobulin superfamily of adhesion receptors. Another important leukocyte-endothelial adhesion protein of the immunoglobulin superfamily is platelet-endothelial cell adhesion molecule (PECAM-1) (gpIIa or CD31).

Cadherins are integral membrane glycoproteins that play important roles in cellular morphogenesis and differentiation as well as cell-cell adhesion. E-cadherins (uvomorulin) are concentrated in the belt desmosome of epithelial cells; N-cadherins are most notably found in the nervous system and P-cadherins are expressed by many different cell-types. (for review, see Abbas AK et al.)[16]

Neuropeptides

In 1987, neuropeptides became an officially recognized entry in the *Index Medicus* at which time there were already 51 different listings. The importance of neuropeptides in the inflammatory response is particularly clear in models of systemic inflammation such as septic shock. As mentioned above, the neural response to tissue injury is one of the body's earliest defense mechanisms. While these substances take part in mediating the early inflammatory response, the net result of their actions depends partly on the relative levels of individual neuropeptides since some appear to be predominantly pro-inflammatory (growth hormone,[17] prolactin,[17] neurotensin,[18] substance P[18]) and others anti-inflammatory (adrenocorticotropin,[17] calcitonin,[18] β-endorphin,[18] somatostatin,[18] α-melanocyte-stimulating hormone[18]). Because neuropeptides act systemically, they are also known to perform a wide variety of physiologic functions. For this reason, inhibition of pro-inflammatory peptides is largely complicated by the appearance of undesirable systemic side-effects. In comparison, inhibition of *locally* acting inflammatory mediators may be considered a more direct approach to anti-inflammatory therapy. (for review, see Aguayo SM et al.)[19]

Biogenic Amines

This group of substances best represents the early phase mediators of the acute inflammatory response. In fact, their fast acting vasoactive properties are immediately evident following their release from activated mast cells, basophils and/or platelets and account for the flushing of the skin commonly seen around the site of injury. The most commonly recognized members of this class are the amines derived from L-tryptophan and L-histidine, serotonin and histamine, respectively. Serotonin is a vasoconstrictor, inhibitor of gastric secretion, neurotransmitter, and platelet activator whose role in inflammatory pathways is primarily

limited to the acute phase. Apart from its high concentrations in the gastrointestinal tract and CNS, large amounts are also stored in the dense granules of platelets and it is this pool in particular which accounts for most of its proinflammatory actions.[20] In terms of anti-inflammatory potential, the success of therapeutic inhibitors of serotonin has fallen short of its anticipated potential. However, various ligands of serotoninergic receptors in the CNS are marketed as anti-emetic, anxiolytic and anti-migraine drugs.

In contrast to serotonin, the clear-cut benefits of anti-histamine therapy illustrate the impact of histamine as a vasodilator, bronchial smooth muscle constrictor and stimulator of eosinophil chemotaxis. Moreover, its role in mediation of the triple response to skin injury suggests that histamine is the earliest proinflammatory mediator to be released during inflammation.[21] At one time histamine was considered to be the most important mediator of the vascular response in acute inflammation. Evidence against such a claim has since confirmed that sustained exposure to histamine leads to a refractory response in as little as thirty minutes following its release. For this reason, antagonists of histaminergic receptors have a narrow therapeutic window, and this represents the greatest limitation in their use as anti-inflammatory agents. (for review, see Aguayo SM et al.)[22]

Complement System

The complement system contributes to almost every cellular process associated with the inflammatory response and, is thus recognized as one of the most potent mediator systems *in vivo*. The majority of the twenty known complement proteins are synthesized in the liver and then released to the systemic circulation, though macrophages are important as local producers of complement molecules at the site of injury. Whether activated by antibodies (classic pathway) or by antigen alone (alternative pathway), the complement cascade fuels a diffuse

number of proinflammatory events. Activation begins with the splitting of protein C3 that can also be accomplished by plasmin and thrombin. Several major actions result, including the stimulation of mast cells and smooth muscle, chemotaxis and opsonization, and formation of a membrane attack complex that destroys target cells by inducing cell lysis (Illustration I-6).

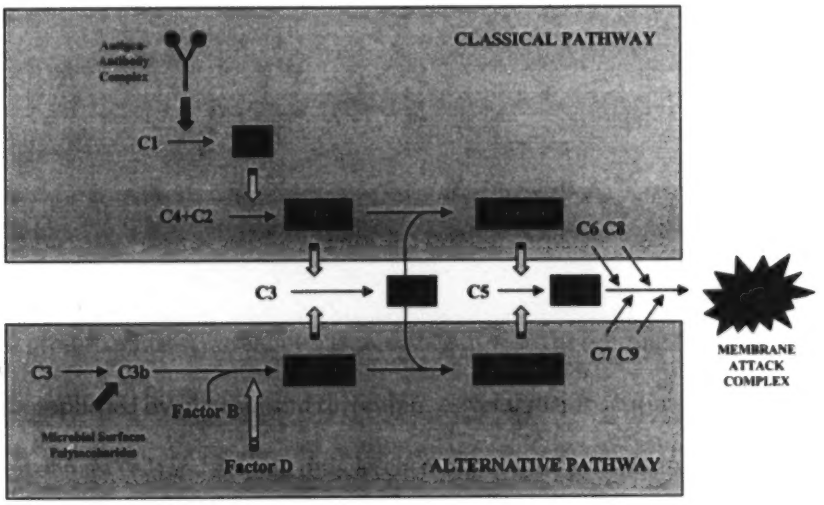


Illustration I-6: **CLASSICAL AND ALTERNATIVE COMPLEMENT ACTIVATION PATHWAYS** Two overlapping pathways of the plasma protein cascade involving complement activation are shown. The classic pathway involves immune complexes or the lipid-A moiety of endotoxin, either of which initate the activation of protein C1 leading to the generation of enzymatically active proteins (shown in boxes). These proteins in turn engage in proteolytic interactions that propogate the cascade. The alternative pathway initially involves the activation of protein C3 by microbial polysaccharides, however subsequent activation steps eventually lead to the same cytotoxic product of the classic pathway- the membrane attack complex.

Many inflammatory diseases have been strongly associated with complement activation, including rheumatoid arthritis, glomerulonephritis, and adult respiratory distress syndrome. Attempts to utilize protease inhibitors as a therapeutic means of blocking complement activation have been largely unsuccessful due to their low degree of selectivity.

Kallikrein-kinin and Blood Coagulation Systems

The kallikrein-kinin system has proven to be extremely important in the initiation and maintenance of inflammation. Under inflammatory conditions, Hageman factor (factor XII of the coagulation cascade) binds to negatively charged surfaces like lipopolysaccharide (LPS), basement membrane or collagen in association with a prekallikrein-kininogen complex. Binding of the trimeric complex successively leads to the activation of Hageman factor, conversion of prekallikrein to kallikrein and high molecular weight kininogen (an α-globulin) to bradykinin [G. bradys, slow + G. kineō, to move, + -in]. Besides acting on kininogens, kallikrein also activates a number of other inflammatory pathways, including MMP-9,[23] plasminogen and complement systems. The pervasiveness of kallikrein activity reflects the multiple actions of kininogens that also include the inhibition of cysteine proteases and part of the acute phase response.

Historically, the term *kinin* refers to a variety of polypeptides, including those originally discovered in snake venom by Rocha e Silva et al.[24] Known to stimulate intestinal and uterine smooth muscle and relax vascular smooth muscle, bradykinin is perhaps the best recognized member of this group. A potent vasodilator, bradykinin is ten times more active than histamine on a molar basis. This is due to its combined direct and indirect actions. Kinins as a group produce direct hypotensive/hyperalgesic effects and many indirect effects through secondary mediators like NO, histamine, prostacyclin, noradrenaline etc.

Several natural inhibitors of kallikreins belong to the group of acute phase proteins including α_2-macroglobulin and α_1-antitrypsin. Degradation of kinins is mainly carried out by kininases. One of these, kininase II, not only removes vasorelaxation but actually generates a pressor effect by converting angiotensin I to angiotensin II, thus known also as angiotensin converting

enzyme. Because kinin degradation is extremely efficient in the lung and kidney, these peptides only reach biologically significant concentrations at the site of inflammation and therefore behave much like autacoids (local mediators). As with histamine, tissues become refractory to the actions of kinins, and therefore, it is unlikely that they exert significant proinflammatory effects beyond the first phase of acute inflammation. Moreover, the effects of kinins clearly depend on a complex cascade of events involving many other mediators. For these reasons, selective inhibitors of this system have not proven useful in the treatment of general inflammatory conditions.

The combined actions of the blood coagulation cascade, platelets, the fibrinolytic system and the tissue repair pathways are responsible for the finely tuned process of vascular haemostasis. Under physiologic conditions the haemostatic process efficiently preserves vascular integrity. Thrombosis is a pathologic extension of haemostasis that involves the formation of a haemostatic plug in an artery, vein or the heart. This undesirable event carries the risk of interrupting blood flow locally or distally through the formation of an embolus. Tissue injury and the ensuing inflammatory response are a good example of conditions that upset the haemostatic balance to flavor activation of coagulation and thrombosis pathways. Reasons for this shift include the advantages of minimizing blood loss from the site of injury and physically isolating the site to aid in the accumulation of responding cells. Activation of the cascade may occur by either of two converging pathways. The extrinsic branch responds to negatively charged phospholipids on activated platelets and factors arising from the injured tissue. The intrinsic pathway commences with Hageman Factor activation as outlined above. Natural inhibitors of the coagulation cascade include antithrombin III, heparin, α_2-macroglobulin and α_2-antitrypsin, many of which are acute phase proteins whose dysfunction or exhaustion may

precipitate disseminated intravascular coagulation. Treatment of coagulopathy secondary to inflammatory disease cannot replace anti-inflammatory therapy, and often the two therapies are combined to treat the acute symptoms along with the underlying disorder. (for review, see Regoli D et al.)[25]

Eicosanoids and Platelet Activating Factor

Early studies of inflammatory pathways were greatly advanced by the work of Sir John Vane and colleagues who in 1971 discovered that acetylsalicylic acid (ASA) and other NSAIDs inhibit the synthesis of prostaglandins and thromboxanes.[26] Since that time, molecular pharmacology has determined the biosynthetic pathway of prostaglandins, thromboxanes, lipoxins and leukotrienes, jointly known as eicosanoids (G. *eicosa-*, twenty, + *eidos*, form). All derived from arachidonic acid, these four classes of mediators may be produced in virtually all cells in proportions determined by the activity or abundance of downstream enzymes. Due to their extremely short half-life in blood, eicosanoids act as local hormones via autocrine or paracrine pathways in response to a variety of chemical or mechanical stimuli. For example, compared with other eicosanoids, thromboxane production is high in platelets and neutrophils while prostacyclin generation is predominant in endothelial cells. Tissue injury provokes a local rise in leukotrienes LTC_4, LTD_4 and LTE_4 from mast cells, an event that contributes to the classic wheal-and-flare response. During the inflammatory response, PGE_2 and PGI_2 are the predominant prostaglandins produced at the wound site where they are known to markedly contribute to local vasodilatation and hyperalgesia. In contrast, these same PGs have been found to down-regulate the chronic inflammatory response by presumably suppressing macrophage generation of TNFα and IL-1.[27] In view of the vast success of NSAIDs as anti-

inflammatory drugs, the potential benefits of drugs that target other branches of eicosanoid synthesis pathways continue to be investigated.

Within the cell, eicosanoid synthesis begins with phospholipase A_2 (PLA_2)-mediated cleavage of arachidonic acid from acyl or alkyl phospholipids found in the phospholipid bilayer (Illustration I-7). Not surprisingly, the unrivalled anti-inflammatory actions of steroids are partly due to their ability to inhibit this fundamental stage of eicosanoid synthesis. In particular they act through the generation of lipocortins, an endogenous protein group of PLA_2 inhibitors.[28,29,30] In the absence of such inhibitors, free arachidonic acid becomes an available substrate for cyclooxygenase (COX; prostaglandin and thromboxane synthesis pathway) or lipoxygenase (lipoxin and leukotriene synthesis pathway). Here again, differences in enzyme distribution have a direct effect on corresponding eicosanoid distribution: lipoxygenase is not as widely distributed as is cyclooxygenase. Of the two forms of cyclooxygenase, constitutive (physiologic) expression is most commonly associated with COX-1, whereas COX-2 is thought to have much more of an inducible activity profile, at least in activated inflammatory cells.

An antipyretic, analgesic and antiplatelet drug, ASA has enjoyed unparalleled success as an over-the-counter alternative to steroid drug therapy by acting as a selective, irreversible COX inhibitor, leaving the synthesis of other eicosanoid metabolites such as leukotrienes, intact. Some critics argue however that certain prostaglandins inhibited by ASA such as prostacyclin and E series prostaglandins may actually produce some desirable anti-inflammatory effects. In partial support of this claim, systemic administration of non-hypotensive doses of these prostaglandins has been found to suppress lymphocyte activation and the release of proinflammatory cytokines from macrophages.[31,32] Since such effects may be potentially useful

in chronic inflammatory disorders like arthritis, the argument remains that treatment of this and other chronic diseases with aspirin or other NSAIDs may not always be beneficial. Adding to this controversy, it now appears likely that the actions and mode of expression of COX enzymes are much more complicated than originally described: COX-1 can exhibit some proinflammatory properties whereas COX-2, which is expressed in high amounts during inflammation and generates excessive amounts of prostaglandins and thromboxane, may to some degree be constitutively expressed.[33] In retrospect, the discovery of COX-2 nevertheless marked a turning point in our understanding of the mechanisms by which NSAIDs produce their therapeutic and toxic effects.[34] Altogether, the pharmacological development of selective COX-2 inhibitors continues to be driven by the concept of increased effectiveness and decreased gastric side effects that are believed to be a major part of the pharmacological profile of this form of anti-inflammatory therapy. Testing of various COX-2 inhibitors continues to show promise though results vary from model to model.[35,36] For many patients suffering from chronic inflammatory diseases, the development of a compound lacking aspirin's gastro-irritant properties remains a much sought after alternative to prophylactic ASA therapy.

Like eicosanoids, platelet activating factor (PAF) synthesis depends on the activity of PLA_2 and so belongs to the second family of autacoids derived from membrane phospholipids. Because the *alkyl* phospholipid required for PAF synthesis quite commonly contains an arachidonyl moiety linked to the second carbon, PAF synthesis typically involves eicosanoid co-production. The actions of PAF are quite distinct from any one eicosanoid: a potent chemotactic agent, PAF is also intimately associated with immune cell priming and the production of pro-inflammatory cytokines like TNF and IL-1. Released by sensitized mast cells following tissue injury, PAF plays an early role in the inflammatory response as a

promoter of platelet activation and vasodilatation. Additionally it is linked to microvascular injury by stimulating eosinophils to release endothelium-degrading substances like major basic protein, LTC_4 and superoxide. Interestingly, the endothelium itself is a rich source of PAF which is produced in response to thrombin, LTC_4 and LTD_4. The unique influence of PAF in the inflammatory response continues to fuel an interest in the development of therapeutically useful antagonists, however, to date no PAF antagonists have become successful as an anti-inflammatory drug. (for review, see Peers SH et al.)[7]

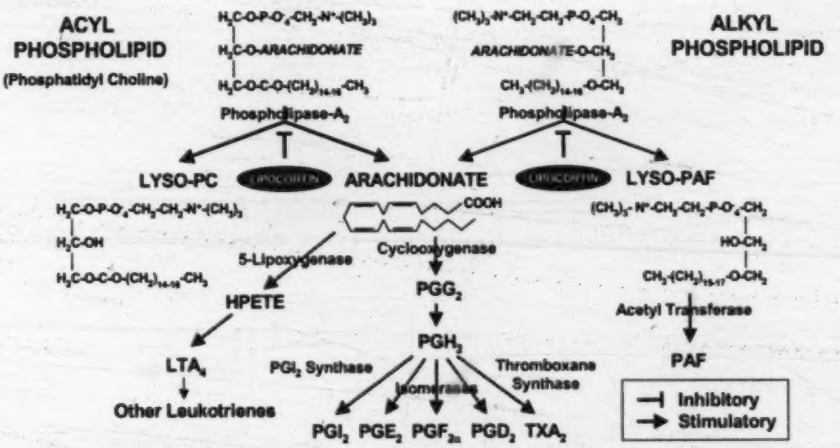


Illustration I-7: **BIOSYNTHETIC PATHWAYS OF LIPID MEDIATORS** Acyl and alkyl membrane phospholipids are readily availableas substrates for phospholipase-A_2 (PLA_2) in various cell types including mast cells. Both substrates generate arachidonate, however the acyl pathway uniquely yields lyso-phosphatidyl choline (Lyso-PC) as opposed to lyso-platelet activating factor, the precursor of PAF (platelet activating factor). As such, the alkyl pathway can produce all 3 classes of lipid mediators: leukotrienes (LT), prostaglandins (PG) and thromboxanes (TX), and PAF, while the acyl pathway does not generate PAF. Lipocortin is a naturally produced inhibitor of PLA_2. Cyclooxygenase and lipoxygenase are key regulatory sites of the PG and LT pathways.

Reactive Oxygen Species

The biogenesis of reactive oxygen species (ROS) by enzymatic and non-enzymatic pathways occurs as a part of many physiologic and pathologic processes. In somatic cells, mitochondrial electron leakage is the greatest contributor to *physiologic* levels of oxygen radicals. Evidence

suggests that excess production of ROS is directly linked to the pathogenesis of many inflammatory disorders.[2] Biological damage produced by ROS may be a consequence of two main events: increased production of ROS and/or decreased levels of natural antioxidants. Several important species of ROS generated *in vivo* include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot}$), peroxynitrite ($ONOO^-$) and the hydroxyl radical ($\cdot OH$). Enzymatic pathways that routinely generate ROS include NADPH oxidase, nitric oxide synthase, uncoupled xanthine dehydrogenase and uncoupled mitochondrial electron transport chains. Non-enzymatic pathways are also important, and these often involve transition metal-catalyzed autoxidation of biomolecules like catechols, quinones, flavins, thiols and haemoglobin. In addition, ionizing radiation may also be an important contributor to rising ROS levels. Cellular destruction can involve lipid peroxidation, protein or DNA oxidation.

A relevant issue surrounding ROS and their contribution to any particular inflammatory disease is the identity of the key participant(s). For instance, though the hydroxyl radical is the most aggressive biological oxidant known, it is typically not produced *de novo*. Looking upstream of the hydroxyl radical however, and recognizing that activated immune cells are a primary source of the ROS generated in inflammation, it is likely that $O_2^{\cdot}$ and nitric oxide (NO) are linked to the oxidative damage that is evident under such conditions. This suggestion however is rather oversimplified since NO has been described as having both pro-oxidant and antioxidant properties. In a recent report addressing this paradox, the biphasic actions of NO were linked to two distinct pathways.[37] At low concentrations NO predominantly engages in peroxidative chemistry that has a protective effect on oxidative killing. Alternately, high concentrations of NO promote cell death by enhancing mitochondrial dysfunction. For superoxide, the situation is different since generation of this

charged species in the extracellular space means that it cannot permeate lipid membranes unless converted to a cell-permeable ROS. Resolution of this state of impermeability however is believed to result from the concomitant upregulation of iNOS and NADPH oxidase in inflammatory cells, an effect placing NO in close proximity to $O_2^{\cdot}$ in proinflammatory microenvironments. Consequently, spatial and temporal generation of these ROS is complemented by their near diffusion-limited ($k = 6.7 \times 10^9\ s^{-1}$)[38] reaction to form $ONOO^-$, a potent oxidant and precursor of the hydroxyl radical .[39]

Cells in their physiologic environment are well equipped with many specific and non-specific antioxidant systems that protect them from the destructive forces of ROS. Some of these include intracellular and extracellular superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione lipoperoxidase, albumin, bilirubin, metal chelators like caeruloplasmin and transferrin, thiols, ascorbate, urate etc. Altogether, spatial, temporal and quantitative aspects of ROS generation are all important determinants of the efficiency of the antioxidant system. Table I-1 below lists the most important sources of ROS along with their corresponding product and its primary anti-oxidant found *in vivo*. (for review, see Winyard PG et al.[40] and Aguayo SM et al.[41])

TABLE I-1

SOURCES OF REACTIVE OXYGEN SPECIES AND THEIR PRIMARY ANTI-OXIDANTS

SOURCE(S)	PRODUCT	1° ANTI-OXIDANT
NADPH Oxidase	$O_2^{\cdot}$	SOD
Xanthine Oxidase	$O_2^{\cdot}$	SOD
$Fe^{2+} + O_2$	$O_2^{\cdot}$	SOD
$Fe^{2+} + H_2O_2$	$\cdot OH$	Non-specific
$NO + O_2^{\cdot}$	$ONOO^-$	Thiols
$SOD + O_2^{\cdot}$	H_2O_2	Catalase
$SOD + ONOO^-$	$\cdot OH$	Non-specific

NOTE: 1° Anti-oxidant = Primary anti-oxidant, NADPH = Reduced nicotinamide adenine dinucleotide phosphate, $O_2^{\cdot}$ = Superoxide, SOD = Superoxide dismutase, Fe^{2+} = Ferrous ion, H_2O_2 = Hydrogen peroxide, NO = Nitric oxide, $ONOO^-$ = Peroxynitrite, OH = Hydroxyl radical

CELLULAR PROTEINASES

Cellular proteinases and their inhibitors profoundly affect the activities of various biochemical pathways that fuel the inflammatory response. Several important groups (described in earlier sections) include the blood clotting cascade, fibrinolytic system, kallikrein-kinin cascade and the complement system. Despite the many types of proteinases known they can all be placed in 1 of 5 classes in which *cysteine*, *serine* (e.g. trypsin, complement factors and components, tissue & plasma kallikreins, thrombin, tissue plasminogen activator, coagulation factors, plasmin), *threonine*, *aspartic* (e.g. pepsin, renin) and *metallo* (e.g. matrixins) groups are central to their catalytic actions. In cysteine, serine and threonine proteinases, a nucleophilic amino acid occupies the catalytic site, whereas the activity of aspartic and metalloproteinases is associated with an activated water molecule that is stabilized by an aspartic residue(s) or zinc, manganese or cobalt atom(s), respectively. Because of these differences, the first three classes of proteinases have the added capability of acting as transferases. Overall, the great variety of these enzymes reflects their broad spectrum of biological functions.

In the course of an inflammatory reaction, mast cells, cytotoxic T-lymphocytes and polymorphonuclear leukocytes become a particularly rich source of proteinases. During acute inflammation for example, polymorphonuclear leukocytes are the first to arrive to the inflammatory site where they release a substantial amount of these enzymes. Macrophages are also an important source of proteinases, acquiring some of their cytotoxic character from an arsenal of cytolytic serine proteases. Because of their prominence in chronically inflamed tissue, macrophages are thought to play an important role in tissue destruction through the release of various matrix-degrading proteases.

In both acute and chronic states of inflammation, matrix metalloproteinases are critical determinants of leukocyte migration and recognition, and thus represent an important subset of pro-inflammatory proteases. As the name suggests, these enzymes degrade components of the extracellular matrix following their release from local cells and immune cells migrating to the site of tissue damage. With degradation of the basement membrane of the capillary wall being one of the earliest steps in the infiltration process, a growing amount of interest has shifted to the type-IV collagenases (MMP-2 and MMP-9) that are responsible for such events. In addition to collagenolysis, the collagenases are capable of degrading many other matrix proteins. Proteoglycan cleavage disrupts articular cartilage integrity while cleavage of glycoproteins alters cell matrix geometry in both bone and cartilage. Notwithstanding their namesake function, matrix metalloproteinases are now known to act on many other important substrates including some cytokines, big endothelin,[42] other proteinases and even some cell receptors. One substrate of particular importance in the developing inflammatory cascade is TNFα. It has been shown that the actions of at least one type of MMP are required for processing of the precursor form of TNFα into its mature form that then is released from

leukocytes.[43] Overall, most of the classic symptoms of inflammation can be attributed to proteinase actions involved in immune cell infiltration, blood product extravasation and cell destruction.

In some instances, a special subset of intracellular proteases and endonucleases actively destroy living cells through the process of apoptosis. Interestingly, such actions are observed during an inflammatory response where they are believed to serve as a regulatory checkpoint of immune cell activity. In contrast to cell necrosis, apoptotic cell death promotes healing of tissue without the release of cellular contents that commonly evoke a secondary inflammatory response. Failure to constrain the inflammatory process through selective immune cell apoptosis could underlie the shift from mildly acute to chronic inflammation or the development of a diverse range of chronic disorders such as asthma, cancer or autoimmune disease.[44] For the many advocates of this hypothesis, the potential use of pro-apoptotic agents to ameliorate these pathologies represents a currently attractive therapeutic strategy.

In blood and tissue fluids that lie distal to the inflammatory site, controlled inhibition of proteinase activity is essential if widespread tissue damage is to be avoided. This is accomplished by non-specific inhibitors like α_2-macroglobulin (an acute phase protein) or specific inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs). Over 70% of anti-proteolytic activity in human plasma can be attributed to the actions of α_1-anti-trypsin.[45] Perhaps the best example to illustrate the biological importance of this enzyme is the autosomal recessive disorder known as α_1-anti-trypsin deficiency. As one of the most common causes of childhood cirrhosis, this disease may also affect the pulmonary architecture and cause emphysema.[46] (for review, see Barrett AJ et al.)[47]

The remainder of this chapter provides a detailed description of two of the proinflammatory mediators mentioned above, i.e. nitric oxide synthase and matrix metalloproteinases. In particular, a short overview of their currently defined role in health and disease is provided, highlighting the work most pertinent to the subject of this investigation. In Parts II and III that follow, the specific background and objectives of the research described in this thesis are respectively outlined for each of two major studies.

Nitric Oxide

Studies of the biology of NO have come a long way since the seminal work of Murad and colleagues[48] who in the late 70's discovered that NO activated soluble guanylate cyclase to raise cyclic guanosine monophosphate levels and produce vasodilatation. Two years later Furchgott and Zawadzki made the seemingly unrelated discovery that the vasodilatory actions of acetylcholine depended on the liberation of what they described as *endothelium derived relaxing factor* (EDRF).[49] In the 1986 Rochester symposium, the connection between EDRF and NO was made when Furchgott and Ignarro proposed that EDRF actually was NO, and that its pathway of generation would likely account for the actions of organic nitrates which British physician Lauder Brunton discovered in 1867 to be effective anti-anginal agents. It was Moncada and colleagues[50] however who were to provide the first experimental evidence showing that the properties of NO accounted for the biological actions of EDRF. For their work, R.F. Furchgott, L.J. Ignarro and F. Murad received the 1998 Nobel Prize in Physiology or Medicine.

As one of the smallest biologically active molecules to be synthesized in the body, nitric oxide (NO) is a gaseous mediator produced from the semi-essential amino acid L-arginine by a family of isoformic enzymes called NO synthases (NOS).[51] Gene cloning has identified at

least three isoforms of NOS: the endothelial type (eNOS; chromosome 7), the neuronal type (nNOS; chromosome 12) and the isoform expressed *de novo* by exposure to LPS or pro-inflammatory cytokines and inhibited by glucocorticoids or a range of other cytokines or growth factors (e.g. TGF-β), and associated with various inflammatory reactions (iNOS; chromosome 17).[51] Overall, these three isoforms exhibit a 50% homology; they are all homodimeric cytochrome P_{450}-type haemoproteins[52] and require calmodulin (and in some cases Ca^{2+}), reduced nicotinamide adenine dinucleotide phosphate, and the prosthetic groups flavin mononucleotide, flavin adenine dinucleotide and tetrahydrobiopterin for normal function (Illustration I-8).

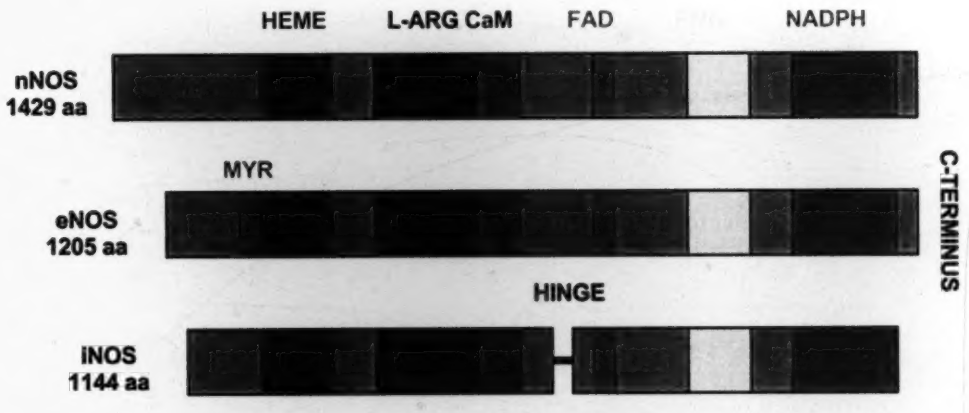


Illustration I-8: **PRIMARY SCHEMATIC SEQUENCE OF NITRIC OXIDE SYNTHASE ISOFORMS** Unlabelled regions are structural. aa = Amino acids. Relative location of binding sites are indicated: HEME = Heme protein, L-ARG = L-Arginine, CaM = Ca^{2+}-Calmodulin, FAD – Flavin adenine dinucleotide, FMN = Flavin mononucleotide, NADPH = Reduced nicotinamide adenine dinucleotide phosphate, MYR = Myristic acid, C-terminus = Carboxy terminal

Apart from requiring its many cofactors, NOS naturally depends on a ready supply of L-arginine and oxygen as reactants. This requirement in turn relies partly on the actions of the Na^+-independent y^+ transport system that is an important regulator of intracellular L-arginine levels.[53] Given their common affinity for L-arginine, NOS and the y^+ transporter may both

serve as candidate targets for the development of inhibitors of NOS. This approach has led to the development of substrate analog inhibitors of NOS. One such example is N^{ω}-monomethyl-L-arginine (L-NMMA), a non-specific NOS inhibitor that interestingly, has been found to be produced endogenously.[54] To date, disease states characterized by increased NO production encourage the development of clinically useful isoform-specific inhibitors of NOS however, none of those reported in the literature has progressed beyond phase III of pharmacological development.

Endothelial (eNOS) and Neuronal (nNOS) Isoforms of NOS

In normal physiology, synthesis of NO by a variety of cell types typically involves the Ca^{2+}-dependent isoforms of NOS (eNOS and nNOS) that participate in the regulation of vascular tone and haemostasis as well as central and peripheral neurotransmission. These diverse processes account for two relatively distinct fields of NO research based on its actions in the cardiovascular system or central and peripheral nervous systems, respectively. Most of the physiologic actions of NO are achieved by virtue of its high solubility in biological fluids; NO freely diffuses across cell membranes and acts via autocrine or paracrine pathways.

As a consequence of its free radical structure, the chemical half-life of NO is only several seconds *in vitro*. In contrast, the biological half-life of NO *in vivo* is believed to be considerably greater due to its reversible reaction with thiols to form S-nitrosothiols.[55,56] In fact, S-nitrosoglutathione (GSNO), the reaction product of NO (or peroxynitrite) and glutathione (the most prevalent non-protein thiol produced in cells) is thought to be a major endogenous form of NO. However, the high reactivity of NO also promotes its rapid inactivation *in vivo* following reactions with heme proteins such as oxyhaemoglobin or oxidants including

superoxide. All pathways of NO degradation ultimately lead to the generation of nitrite and nitrate, the common end-point molecules of its metabolism.

Under physiological conditions it appears that mechanical stimuli like pulsatile flow and shear stress stimulate the continuous (basal) release of NO by eNOS. Moreover, physiologic stimulation of endothelial cell receptors by acetylcholine, bradykinin or a number of other biomolecules has also been suggested to activate eNOS leading to the generation of NO.[57] The exact mechanism responsible for regulation of basal eNOS activity is not known, however the process may begin with the transient production of an intracellular calcium spike that activates NOS. The bioactivity of NO that follows depends upon its reactions with affinity targets such as the soluble guanylate cyclase (Illustration I-9).[58]

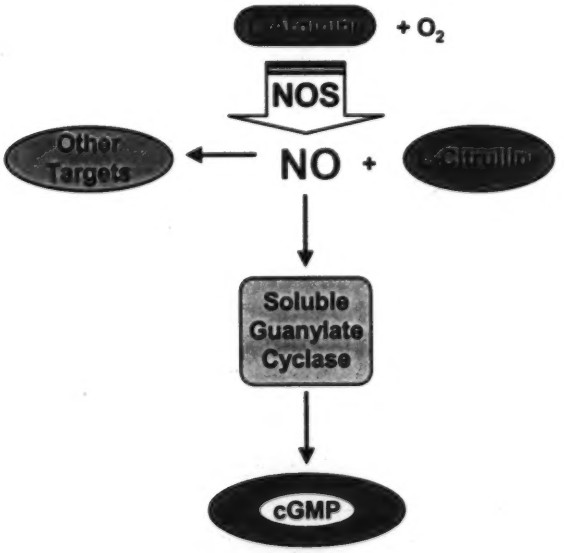


Illustration I-9: **NITRIC OXIDE SYNTHESIS** The amino acid L-arginine is a substrate for all 3 isoforms of nitric oxide synthase (NOS), which, in the presence of other co-factors is hydrolyzed to form nitric oxide (NO) and L-citrulline. Once generated, NO will readily diffuse into the extracellular space where it may oxidize various target molecules in or around nearby cells. An important pathway of NO reactivity involves its stimulation of soluble guanylate cyclase. This leads to the generation of cyclic guanosine monophosphate which results in activation of a cellular protein kinase and the cascade of associated effects.

To regulate vascular reactivity, NO readily diffuses from the endothelium and enters adjacent vascular smooth muscle cells where it binds to and activates soluble guanylate cyclase leading to a rise in intracellular cGMP and a subsequent fall in intracellular calcium levels. This in turn leads to relaxation of the smooth muscle cells and vasodilatation.[59] In addition to these actions, NO has also been shown to inhibit vascular smooth muscle proliferation, and this ability has a significant impact on vascular homeostasis. Overall, the ability to produce a profound vasodilatory effect is a hallmark of NO physiology (so called "vasodilator tone"), thus establishing it as a critically important regulator of vascular tone throughout the body (Illustration I-10).

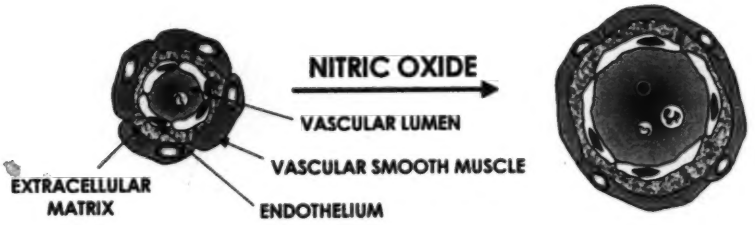


Illustration I-10: **ENDOTHELIUM DEPENDENT VASCULAR RELAXATION** Vascular tone is dynamically regulated by changes in the balance of vasoconstrictors and vasorelaxants. This balance is altered during the inflammatory process with the net effect being vasodilatation. Proinflammatory cytokines like tumor necrosis factor (TNF) stimulate the production of vasodilators like nitric oxide (NO) and prostacyclin (PGI_2). This results in relaxation of the vascular smooth muscle and endothelial lining, thus facilitating the enhanced delivery of leukocytes to the inflammed tissues.

Nitric oxide also enters the blood stream where its diffusion into platelets is facilitated by the shear forces that drive platelets into a slow moving stream near the endothelium,[60] thereby making them readily accessible targets for NO. Moreover, NO is produced by activated platelets, thus having both autocrine and paracrine effects on platelet action. The net result accounts for another important biological action of NO- inhibition of platelet adhesion and aggregation. This property makes NO an important regulator of vascular homeostasis.

Perhaps the most unconventional role for NO is that of a neurotransmitter or more likely a neuromodulator. In this case we see the involvement of a distinct Ca^{2+}-dependent isoform of NOS (nNOS), which is predominantly expressed in discrete neuronal populations in the body and unlike eNOS, is not membrane bound by a myristoyl moiety. Regulation of nNOS activity is acutely managed via allosteric effects, posttranslational modification and subcellular redistribution while long-term modifications result from changes in gene transcription.[61] In the CNS for example, release of glutamate from a firing excitatory neuron leads to the stimulation of post-synaptic NMDA receptors causing a rise in intracellular calcium. This activates Ca^{2+}-dependent nNOS and NO is released to affect surrounding neurons in an anterograde or retrograde fashion. In fact, these actions of NO have been suggested to influence neuronal development and memory formation through both long-term synaptic potentiation and depression. It appears that excessive NMDA receptor stimulation and nNOS stimulation may underlie the cellular damage detected in ischemic stroke, epilepsy, Huntington's disease and Parkinson's disease.

In the peripheral nervous system, nNOS is most abundant in nonadrenergic-noncholinergic (NANC) neurons associated with the gastrointestinal and genitourinary tracts, pulmonary epithelium, renal macula densa and pancreatic islets. Throughout the GI tract, NO produces a dilator tone that is absent in some gastrointestinal disorders such as achalasia.[62] Furthermore, it is thought that disturbances in NOS activity in autonomic nerves is linked to various pathophysiological conditions including migraine headache, hypertrophic pyloric stenosis and male impotence.[61]

Beyond its multifunctional role in human physiology, the salutary actions of NO generated by eNOS in the pathological setting are intriguing. At basal levels (pmol-nmol), NO generation

has also been shown to exert anti-inflammatory effects through its combined abilities to target inflammatory cells and platelets (Illustration I-11). Under these conditions NO is capable of inhibiting the synthesis of eicosanoids PGE_2 and TXB_2,[63] IL-6[64,65] and superoxide;[66] adhesion of leukocytes;[67] and the degranulation of mast cells[68] and platelets.[51] Though many of these actions likely involve secondary mediators such as cGMP or cGMP-dependent protein kinase, the mechanism by which NO suppresses eicosanoid generation is believed to involve its direct interaction with the heme moiety of COX-2.[63] Apart from these observations however it is important to recognize that NO has also been shown to induce prostaglandin production.[69] In view of this paradox, it appears that the interactions of NO with eicosanoid generation may be cell-dependent.

A significant body of literature characterizing eicosanoid pathways comes from the work of JL Wallace and colleagues. From their investigations a new class of anti-inflammatory drugs has emerged.[70] By coupling a nitric oxide-releasing moiety to NSAIDs, NO-NSAIDs appear to be much less ulcerogenic than the parent NSAID when it is used alone.[71] Indeed the gastro-protective effects of NO have been known for over a decade.[72] Mechanistically, NO is thought to counteract the reduction in mucosal perfusion and the rise in neutrophil infiltration that are suspected to contribute to NSAID toxicity while at the same time reducing neutrophil infiltration into the inflammatory lesion.[73,74] Though studies are still preliminary, the therapeutic usefulness of this new class of compounds appears quite promising.

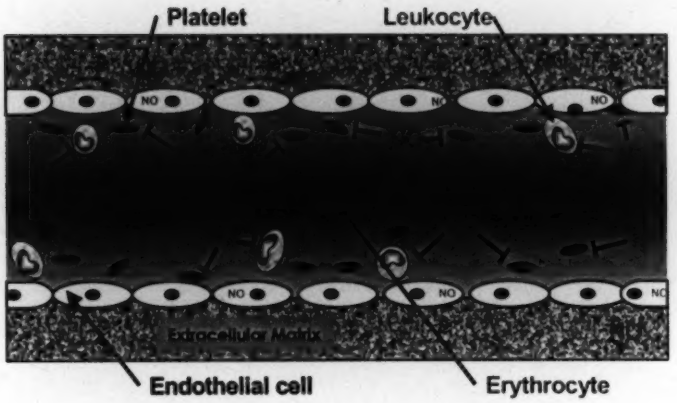


Illustration I-11: **NITRIC OXIDE AS AN ANTI-INFLAMMATORY AGENT** The inflammatory process leads to the activation of many cell types that respond by producing large amounts of nitric oxide (NO). Platelets and endothelial cells are shown here as sources of NO that has a direct effect on other cells in the vicinity. As an anti-inflammatory agent, NO inhibits platelet activity and leukocyte binding to the endothelium. In opposition to these effects, local concentrations of NO may be rapidly depleted as NO penetrates erythrocytes to oxidize hemoglobin.

Thus, physiologic generation of NO by eNOS and nNOS is of paramount importance to maintain body homeostasis. Therefore, it is not surprising that impaired synthesis of NO has been implicated in the pathogenesis of various vascular pathologies including atherosclerosis, hypertension, diabetes and pre-eclampsia.[75,76]

Inducible NOS (iNOS)

In contrast to eNOS, iNOS expressed in many cells following pro-inflammatory cytokine stimulation is capable of generating high amounts of NO over long periods of time. These excessive amounts of NO may be potentially toxic. In this section I will now describe how high output of NO may contribute to the pathogenesis of the inflammatory lesion.

Key stimuli responsible for the expression of iNOS have been demonstrated in many different cell types including human hepatocytes and they include LPS or one or more of the

proinflammatory cytokines IL-1β, TNFα, and IFN-γ.[77] Results of molecular cloning studies of the human iNOS gene suggest that a binding site for the transcription factor nuclear factor-κB (NFκB) is an important component of the response element associated with the various mediators of iNOS induction.[78,79] Circulating throughout the body, the above cytokines stimulate a wide variety of cell-types to express a Ca^{2+}-independent isoform of NOS (iNOS).[80] Conversely, anti-inflammatory cytokines liberated during the Th2 response, including TGF-β, IL-4 and IL-10 are known for their ability to inhibit iNOS induction.[81,82] Moreover, NO itself is a feedback inhibitor of NOS. From these and many other findings, a third major field of NO research has evolved dealing specifically with the actions of NO in immunologic and inflammatory processes.

First discovered in activated macrophages, iNOS characteristically generates high local concentrations of NO. At these heightened levels, NO will overwhelm normally tolerant cells by reacting with a wide range of cellular targets and in some micro-environments this yields a cytotoxic effect. Under inflammatory conditions, NO will readily encounter and react with superoxide resulting in the formation of peroxynitrite (ONOO-), a potent and aggressive oxidant.[39] The cytotoxic potential of NO or its metabolites ultimately rests on the degree of spatial and chemical susceptibility of cellular targets such as haem groups, Fe-S or Zn-S clusters and sulfhydryl moieties, all of which may be found in many important metabolic enzymes. These include mitochondrial complexes I and IV, aconitase and ribonucleotide reductase. By reversibly binding to cytochrome c oxidase (complex IV), NO demonstrates some capacity as a regulator of cellular respiration. In contrast to these modest actions, the toxicity of peroxynitrite is exemplified by its irreversible inhibition of complexes I and III.

The proinflammatory actions of NO are apparent throughout all phases of the inflammatory response. Beginning right from the moment of injury, NO generation by local mast cells contributes to the regional vasodilatory effect underlying the symptom of redness. Moreover, the early immune response to inflammatory stimuli is characterized by the infiltration of activated immune cells that are known to generate ROS and NO as a chemical means of destroying foreign or dysfunctional cells. Apart from its direct involvement in inflammation, NO exerts an indirect influence through other pathways. Recent studies have uncovered a cross-talk pathway in which NO enhances the activity of cyclooxygenase leading to the generation of excessive amounts of proinflammatory prostaglandins.[83]

The impact of high-output NO generation on proinflammatory pathways has also been interpreted clinically. The proinflammatory profile of NO generated by iNOS indicates that inducible NO plays an important role in the pathogenesis of various acute and chronic inflammatory diseases. One of the most compelling examples of acute inflammatory actions of NO is septic shock. Numerous studies have shown that large amounts of NO are generated during septicaemia.[84,85] This NO may be responsible in part for the vascular paralysis, microvascular collapse, myocardial dysfunction and decreased cellular respiration that characterize this disease. Indeed, studies using the iNOS knockout mouse have shown that they are less susceptible to the septic insult when compared to the wild type mice.[86] There are also some examples of inducible NO acting in the setting of chronic inflammatory reactions that cause tissue and organ destruction. Inflammatory arthritis,[87] hyperoxia-induced lung injury[88,89] and myocardial injury following myocarditis[90] are chronic pathologies in which inducible NO has been implicated as one of the pathogenetic factors.

Interestingly, some researchers indicate that not all actions of inducible NO that take place during inflammation may be detrimental to the host. Studies with inhibitors of NOS showed that inflammation-induced organ injury could be exaggerated following the inhibition of this enzyme.[91,92] However, interpretation of these findings is confounded by the fact that the experiments that evidenced protective effects of inducible NO were performed using isoform-non-selective NOS inhibitors that also decreased the protective effects of NO synthesized by eNOS. I will return to this discussion of injurious vs. protective effects of inducible NO in the chapter on chronic liver disease.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) constitute a large family of ubiquitous zinc-dependent extracellular matrix degrading and remodeling enzymes.[93,94] They are synthesized as latent enzymes (proMMPs) that are stored in intracellular granules and released to the extracellular environment upon cell stimulation where they may degrade any of about 140 known matrix proteins. Illustration I-12 identifies the most distinguishing common domain features of major members of the MMP family. Within the C-terminal domain are Cys516 and Cys704, conserved in all metalloproteinases that contain a 4 repeat C-terminal hemopexin-like domain that is important for TIMP binding. A conserved Cys99 plays an essential role in maintenance of the proenzyme state of MMP-9 by interacting with the zinc ion of the active centre, and is cleaved off upon enzyme activation. A unique insert found in gelatinases (MMP-2 and MMP-9) consists of three tandem repeats of the type II module found in fibronectin and cattle seminal fluid. Functionally, both the hemopexin and fibronectin domains serve as important exocites associated with substrate binding.

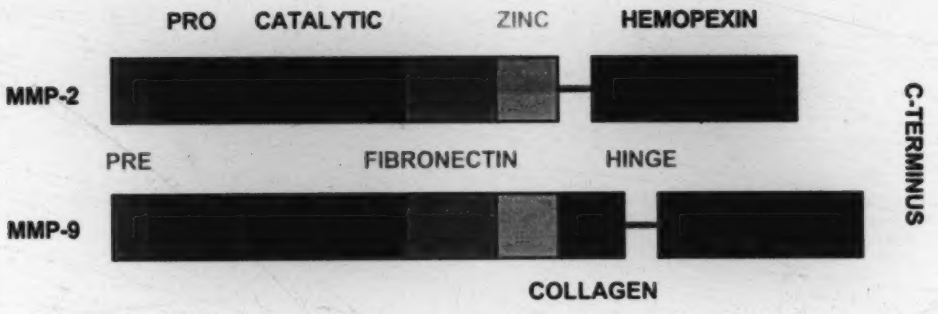


Illustration I-12: **MMP-2 AND MMP-9 FUNCTIONAL DOMAINS** The relative location of some functinal domains and binding sites are indicated for two key members of the gelatinase subfamily of matrix metalloproteinases (MMP-2 and MMP-9). The fibronectin, hemopexin and collagen domains are involved in substrate binding. PRE = Pre-activation exo-moiety, PRO = Pro-activation endo-moiety, C-Terminus = carboxy terminal. Cleavage of PRE and PRO moieties yields the fully active species.

Classified on the basis of function, gelatinases were first recognized for their affinity to a unique variety of cell matrix proteins including gelatine (denatured interstitial collagens), collagen types IV, V, VII, X and elastin.[95] Nowadays, the implications of gelatinases in pathology go far beyond their ability to degrade matrix proteins. Recently it has been shown that *both* MMP-2 and MMP-9 can proteolytically activate latent TGF-ß.[96] Moreover, MMP-2 has been shown to cleave big-endothelin-1 yielding a novel and potent vasoconstrictor, medium endothelin-1.[42]

Once they are activated, matrix metalloproteinases are regulated by non-specific (e.g. α_2-macroglobulin) and specific tissue inhibitors of metalloproteinases (TIMPs). The inhibitory actions of α_2-macroglobulin require that it bind with MMPs and subsequent undergo clearance from circulation, whereas the TIMPs act by tightly complexing ($Ki < 100$ pM)[97] with MMPs and thereby impairing their catalytic activity. In health, MMPs participate in various physiologic processes such as embryogenesis, angiogenesis, bone resorption, wound healing and modulation of platelet aggregation.[98,99]

Due to the presence of a constitutive promoter gene,[100] MMP-2 is ubiquitously expressed and highly dependent on post-translational regulation. When secreted as a latent enzyme, proMMP-2 may be activated extracellularly by other MMPs that proteolytically cleave the propeptide occupying the active site cleft. Extensive work has also revealed a more sophisticated pathway of MMP-2 activation involving the tandem actions of TIMP-2 and the largest known subfamily of MMPs, membrane type MMPs (MT-MMPs). Located within the membrane and extending into the cytoplasm, MT-MMPs are common to many cell types.[101] *In vitro* studies have shown that TIMP-2 acts as a chaperone to cytosolic proMMP-2 by promoting its association with MT- MMP via formation of a ternary complex followed by the release of activated MMP-2.[102,103] In comparison, proMMP-9 is secreted from cells in a stable complex with TIMP-1 at which point it is believed to be activated extracellularly by a variety of agonists including plasmin, reactive oxygen species (ROS), and MMP-3 (Illustration I-13).[104]

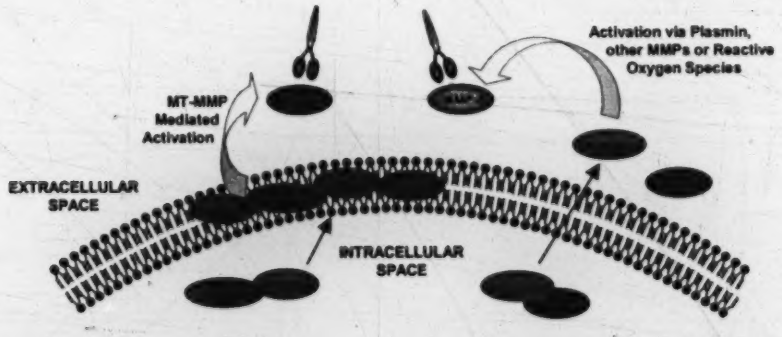

Activation via Plasmin, other MMPs or Reactive Oxygen Species
MT-MMP Mediated Activation
EXTRACELLULAR SPACE
INTRACELLULAR SPACE

Illustration I-13: **GELATINASE ACTIVATION PARADIGM** Some of the primary pathways involving the processing of mature, active forms of matrix metalloproteinases MMP-2 and MMP-9 are shown above. Within many different cell types, the latent form of MMP-2 (proMMP-2) is sequestered by a cytosolic *tissue inhibitor of metalloproteinases* (TIMP) to the cell membrane where its interaction with a *membrane-type MMP* (MT-MMP) leads to its proteolytic activation and release from the cell. In contrast, the latent form of MMP-9 (proMMP-9) is thought to depend, in part, on a temporary interaction with a cytosolic TIMP that promotes its secretion from the cell, often remaining in its latent form. Activation of proMMP-9 will occur by a variety of reactions involving plasmin, other MMPs or reactive oxygen species.

In general, membrane-bound MMP-2 is believed to participate in normal collagen turnover by fragmenting fibrils prior to phagocytosis.[105] Changes in MMP-2 expression have however been noted under some disease conditions. *In vitro*, membrane-bound MMP-2 has been detected in the invadopodia of some tumour cell lines and therefore has been linked to cellular invasiveness.[106] As a pro-inflammatory agent, MMP-2 is expressed by human endothelial cells following their exposure to sublethal levels of H_2O_2.[107] This finding therefore identifies activated endothelial cells as potential contributors to the basement membrane degradation that is commonly invoked by invading immune cells.

The feasibility of measuring the levels of MMPs in blood has been demonstrated in a rat model of ischemia-reperfusion injury where the acute release of MMP-2 from the myocardium antagonized the subsequent recovery of cardiac mechanical function.[108] In critically ill neonates suffering from hypoxic respiratory failure, treatment by extracorporeal membrane oxygenation (ECMO) was associated with a significant increase in the plasma level of MMP-2.[109]

Perhaps the most distinguishing difference between MMP-9 and MMP-2 is the element of inducibility. Unlike MMP-2, the gene for MMP-9 contains a number of classic elements characteristic of inducible genes including a TATA box and AP-1 motif.[110] The induction of MMP-9 takes place in T-cells and macrophages in the presence of some growth factors such as platelet-derived growth factor (PDGF) and proinflammatory cytokines including TNFα and IL-1.[111,112] Moreover, like iNOS, induction of MMP-9 by these cytokines requires the presence of NFκB. In contrast, TGF-β, IFN-β, IFN-γ, progesterone and corticosteroids are known to down-regulate the induction of MMP-9.[113,114,115] Interestingly, there appears to be some degree

of cross-talk between MMPs and NO. In rat mesangial cells stimulated with IL-1β, the addition of NO donor resulted in a significant reduction in mRNA levels of MMP-9.[116]

Increased expression and activity of MMP-2 and MMP-9 have been implicated in a variety of disorders that are linked to acute and chronic inflammatory migration of effector cells. Indeed, MMP-9 overproduction has been linked to atherosclerosis,[117] abdominal aortic aneurysm,[95,118] adult respiratory distress syndrome,[119] arthritis,[120] multiple sclerosis[121] and enhanced tumour cell invasiveness and metastatic potential.[122] In rats suffering from inflammatory bowel disease, a marked reduction in the extent of tissue injury and inflammation (myeloperoxidase activity) resulted with 7 days dosing of marimastat, a broad spectrum inhibitor of MMPs.[123] Despite the abundance of reports describing pathologic changes in MMPs derived from a specific source tissue, only recently has a growing interest in the significance of *circulating* MMPs become apparent. Recently, plasma MMP levels were measured in humans following their injection with endotoxin. Results revealed that polymorphonuclear leukocytes are responsible for a rapid rise in plasma proMMP-9 that might therefore serve as an early marker of cell activation.[124]

RESEARCH OBJECTIVES

STUDY I: CHRONIC INFLAMMATORY LIVER DISEASE

The progression from chronic liver disease to end-stage status is often heralded by systemic sequelae such as hyperdynamic circulation and impaired response to vasoconstrictors, suggesting the involvement of vasoactive mediators with a pro-inflammatory profile. Over the past ten years it has become increasingly apparent that nitric oxide (NO) may be a candidate for such a mediator.[125] In the liver, the ceaseless demand for ammonia detoxification means that urea cycle enzymes like arginase are highly active. Because arginase, like NOS, is an L-arginine dependent protein, the cross-talk between these two enzymes may be important in the progression of chronic liver disease. Most of the available data on the role of NO in liver function is derived from *in vitro* experiments, as well as from various animal models of liver disease. Very little information has been generated concerning the expression and localization of NOS in healthy and diseased human liver. The first research objective of this thesis is to study the expression of NOS and arginase in surgical sections of normal and diseased human liver.

The concomitant changes in the extracellular matrix turnover that accompany liver disease are largely due to local changes in the activities of matrix metalloproteinases which are poorly understood. Members of the gelatinase subfamily such as MMP-2 and MMP-9 are particularly important in such processes due to their unique substrate specificity and pro-inflammatory properties. In the last few years the measurement of plasma MMP levels has gained a considerable amount of interest due to its potential use as a marker for the diagnosis and monitoring of the clinical course of disorders associated with inflammation and injury. With

respect to human liver disease however, only a few studies have measured plasma MMPs in patients with a limited variety of liver disorders.[214,225,227,228] More importantly, no reports have been published so far to compare plasma MMP levels in patients suffering from a wide range of liver diseases to those determined within their respective liver tissue. Therefore, the second research objective of this thesis is to measure the expression of MMP-2 and MMP-9 in surgical sections of normal and diseased human liver.

The third research objective of this thesis is to measure the activity of NOS and MMP systems in samples of peripheral blood obtained from patients undergoing liver transplant for end-stage liver disease. This will serve to correlate the data on NOS and MMP expression in the diseased organ with that in the systemic circulation and also to analyze the impact of liver transplant on these enzymes.

STUDY II: ACUTE INFLAMMATORY DISEASE

An acute inflammatory response to the cellular injury is orchestrated by pro-inflammatory cytokines, a network of host communication and effector proteins.[126] Interleukin-6 (IL-6) is a multifunctional cytokine that plays a central role in host defense due its wide range of immune and hematopoietic activities and its potent ability to induce the acute phase response.[127]

Many of the pro-inflammatory effects of cytokines are attributable to the generation of downstream mediators including nitric oxide synthase (NOS)[51] and matrix metalloproteinases (MMPs).[93,94,128] Cytokine-dependent expression of inducible NOS (iNOS) generates large amounts of NO that contribute to the inflammatory injury.[80] Similarly, excessive expression of MMPs has been implicated in the pathogenesis of inflammatory states including acute respiratory distress syndrome[119] and rheumatoid arthritis.[120]

Although evidence for the role of IL-6, NO and MMPs in the pathogenesis of inflammatory lesion in various animal models is clear, much less is known about the expression and role of these mediators in humans, in particular, in critically ill patients admitted to the intensive care unit (ICU).

The first objective of this study was to measure plasma levels of NO_x^-, IL-6, MMP-2 and MMP-9 in a group of patients hospitalized because of life-threatening conditions. All these patients were admitted to the Pulmonary Intensive Care Unit of the University of Alberta Hospital with respiratory failure including acute lung injury and inflammation.

The second objective was to correlate the admission levels of NO_x^-, IL-6, MMP-2 and MMP-9 with the clinical index of disease severity (APACHE II score) determined for each patient upon admission to the ICU.

Chapter II

CHRONIC INFLAMMATORY LIVER DISEASE

Liver disease is the fourth leading cause of death in Canada. Since the first successful liver transplant was performed in the late 1960's,[129] advances in surgical techniques, organ preservation, medical management and immunosuppressive therapy have made orthotopic liver transplant (OLT) a much more reliable means of therapy. In terms of morbidity, chronic infection with the hepatitis C virus is the most common illness requiring OLT. Nevertheless, OLT is also useful in treating acute fulminant hepatic failure as well as a variety of chronic liver diseases, including: hepatitis; cirrhosis; cholestatic disease; metabolic disorders; Budd-Chiari syndrome; and select intrahepatic malignancies. Unfortunately, a shortage of donor organs often means that the condition of many patients awaiting OLT will progress to a state of end-stage liver disease, a devastating condition hallmarked by diffuse inflammation and parenchymal necrosis. In such instances, despite a large pharmacologic effort to develop safer and more effective anti-inflammatory drugs, the treatment of advanced liver disorders, at best, can only delay the progression of patients to the point of "no return" i.e., liver failure. **Therefore, the understanding of pathogenetic mechanisms that lead to liver failure is of great importance for the development of more effective liver-protective drugs.**

LIVER ANATOMY AND PHYSIOLOGY

To appreciate the profound impact that diseased liver has on normal biological function, a basic understanding of liver anatomy and physiology is required. As the largest single internal organ in the body, the average adult liver weighs 1200-1800 grams. Blood supply to the liver

comes from two sources: the hepatic artery and the portal vein, the latter carrying nutrient-rich blood rich from the stomach, intestines, pancreas and spleen. Each minute the portal vein delivers over 1 litre of blood to the liver, almost 3 times that of the hepatic artery. While blood volume reaching the liver may be relatively constant, the flow ratio in the portal vein vs. the hepatic artery is quite dynamic. Intuitively, such versatility could impact on liver metabolism and therefore receives further discussion below. In the liver, arterial and venous vessels pass through a portal canal from where they drain into endothelial-lined fenestrated capillaries called sinusoids.

The arrangement of six radially aligned sinusoids leading to a common central vein comprises the liver *lobule*. Measuring only a few millimeters in diameter, liver lobules are surrounded by a modicum of connective tissue and account for the overall mass of the liver. It is here that the parenchymal cells of the liver (hepatocytes) extract nutrients from the blood in exchange for waste products in order to carry out a complex array of metabolic functions. For example, bile is produced by hepatocytes from where it is transferred, via canaliculi, into intrahepatic bile ducts that also line the portal canal. Located outside of the liver, the extrahepatic bile tract includes the gallbladder; a receptacle specialized in bile concentration and storage. Histological examination identifies hepatocytes as the major cellular source of liver parenchyma (Illustration II-1). Hepatocytes are polyhedral, polarized epithelial cells that contribute to over 80% of total lobular cellularity.[130] In the single-cell liver plate, the three surfaces of hepatocytes comprise disproportionate amounts of total cell area: sinusoidal (72%); canalicular (13%) and intercellular (15%).[131] Also located within the liver lobule are a number of specialized cells including natural killer lymphocytes (Pit cells), resident phagocytes (Kupffer cells) and fat

containing Ito cells. Smooth muscle cells also form part of the hepatic non-parenchymal cell population though they are not a component of sinusoidal vessels.

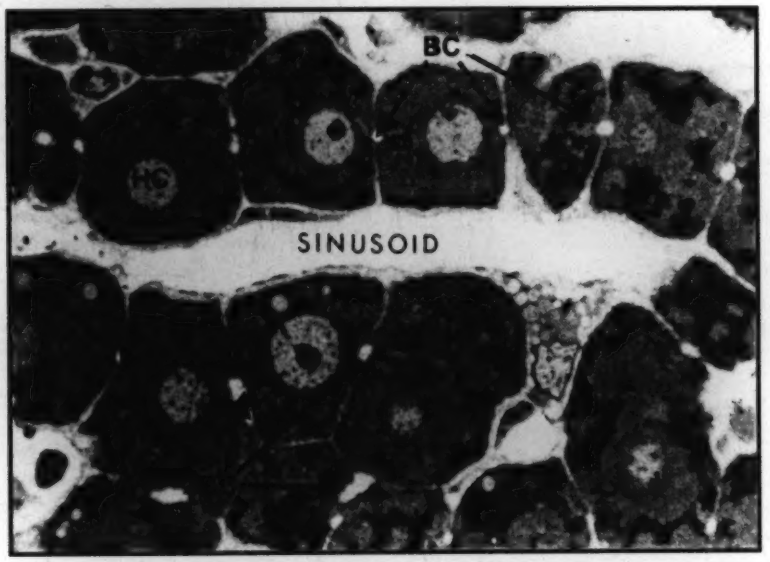


Illustration II-1: **LIVER HISTOLOGY** This micrograph of healthy liver tissue shows the abundant distribution of hepatocytes (HC) in a section of hepatic lobule. Also visible are the biliary canaliculi (BC) though which bile is transported out of the lobule, and the hepatic sinusoid, through which blood perfuses the lobule in its course toward the central vein.

The liver has an extensively developed lymphatic system that serves as the single largest source of lymph in the body, producing 15-20% of total lymph volume. Resistance to portal flow is greatest in the liver, though normally quite low overall (4-8 mmHg). While parenchymal blood supply is dynamically regulated by the terminal hepatic arterioles, distension of the vascular tree in response to an increase in portal flow serves to keep portal pressure within a normal range. Both sympathetic and parasympathetic branches of the autonomic nervous system course the liver along the portal tract. Modulation of hepatocyte and perisinusoidal cell function is largely accomplished by sympathetic tracts in parenchyma along the sinusoids. In particular, carbohydrate metabolism is fine-tuned in hepatocytes whereas neurally-induced

contractions of perisinusoidal cells regulate intrasinusoidal blood flow.[132] Despite the fact that sinusoidal endothelial cells and Kupffer cells also contain contractile proteins,[133] it is the Ito cells that colocalize with areas of greatest luminal contraction.[134] Following passage through the sinusoids, blood enters the central vein and flows to one of three valveless hepatic veins that anastomose with the inferior vena cava, completing the hepatoportal leg of its circulation (Illustration II-2).

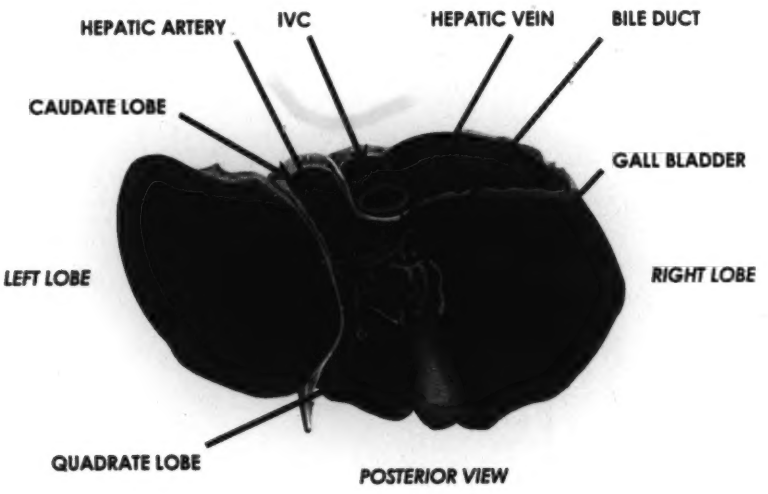


Illustration II-2: **SKETCH OF HUMAN LIVER ANATOMY** The main anatomical structures and morphology of human liver is shown above in a posterior view. The four lobular regions include the lateral left and right lobes and the medial caudate and quadrate lobes. Also shown is the hepatic artery, the inferior vena cava (IVC), the hepatic vein, bile duct, and gall bladder.

Despite similar histological appearance there is growing evidence that hepatocytes localized in distinct regions of the liver may be functionally different from each other. Therefore, the concept of intrahepatic cellular heterogeneity deserves special mention. Investigations into the morphologic, biochemical and functional heterogeneity of hepatocytes have greatly advanced with modern laboratory technology, though this phenomenon itself can be dated back to 1846.[135] In 1958, the term acinus was first used to describe a more precise structural and

functional microvascular unit of the liver parenchyma.[136] The unit, known also as Rappaport's acinus, encompasses all the hepatocytes located in two adjacent lobule segments that are supplied by a terminal branch of the portal vein and hepatic artery and lie between two terminal hepatic venules (Illustration II-3).

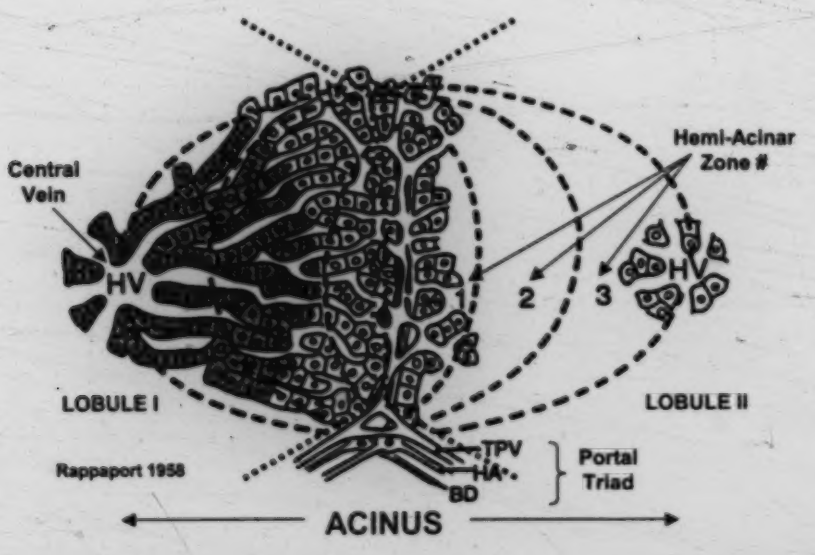


Illustration II-3: **RAPPAPORT'S ACINUS** The sublobular region of the liver described by Rappaport is known as the hepatic acinus. This area spans the region between two adjacent central veins (HV) that are associated with the same portal vein (TPV), hepatic artery (HA) and bile duct (BD). The acinus may be considered as 2 hemi-acini, each of which is subdivided into 3 major zones of heterogeneity: numbering 1-3 from the portal triad toward the HV. As such, hepatocytes in zone 1 (lightest shading) are exposed to the highest blood solute concentration relative to zone 3 hepatocytes (dark shading).

Accordingly, the acinar region associated with a single lobule is known as a hemi-acinus and can be seen in Illustration II-4 below. An important connotation to the acinar model is that blood flow in the acinar sinusoid is unidirectional and that solute exchange between blood and hepatocytes occurs sequentially. The resulting variations in blood composition along the acinar sinusoid produce a state of microenvironmental heterogeneity to which hepatocytes are exposed.

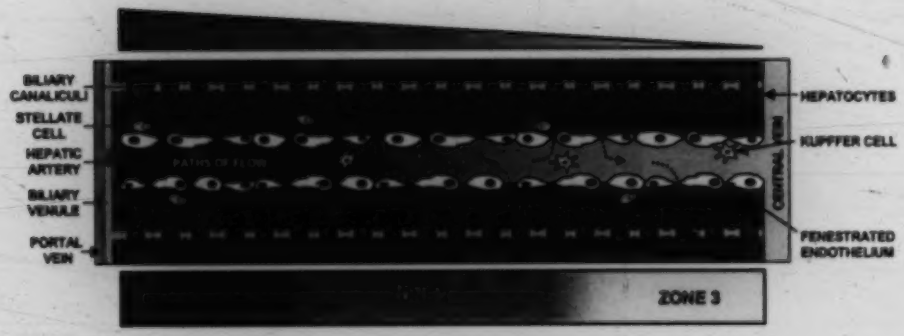


Illustration II-4: **HEPATOCELLULAR HETEROGENEITY OF THE HEPATIC HEMI-ACINUS**
The unidirectional flow of blood through the hepatic hemi-acinus has a great impact on its composition from zone 1 to 3. At zone 1, near the portal triad, the concentration of solutes is highest, and thus the cells bordering this region receive the earliest opportunity to absorb nutrients etc. This is especially important for the parenchymal cells since these cells are especially active for normal liver function. The various diversions in blood flow that are shown above allude to the many sites that may remove essential nutrients and reduce their availability for cells located downstream toward the central vein.

Supporting evidence is now quite abundant, having gained momentum in the late 50's and early 60's with histochemical and enzymology studies performed by various groups.[137,138] Building on evidence for enzymatic zonation, studies spanning a decade described groups of zoned enzymes, giving rise to the concept of metabolic zonation.[139,140] A number of important aspects however must be considered when discussing metabolic zonation: location and activity of the rate limiting enzyme; availability of substrates, cofactors, hormones, oxygen and other regulators. Furthermore, hepatic nerve regulation may affect patterns of acinar blood flow.[141,142] In general, microenvironmental heterogeneity and zonal gene expression very early in life are believed to underlie the development of hepatocyte heterogeneity. Some of the earliest and most complete studies of metabolic zonation investigated carbohydrate metabolism. These studies showed that the glucagon-sensitive glucose-forming pathways such as glycogenolysis and gluconeogenesis were seen in periportal hepatocytes whereas perivenous hepatocytes housed insulin-sensitive glucose-consuming pathways, i.e. glycogen synthesis and

glycolysis.[139,140,143,144,145]

Another case of metabolic zonation that is a subject of greater focus herein is the process of the hepatic urea cycle. Evidence shows that all five urea cycle enzymes are predominantly localized to zone 1 and thus constitute the major site responsible for urea synthesis.[146] Theoretical modulators of zonal heterogeneity have been proposed for both zone 1 and 3, namely mesothelial cells and endothelial cells of hepatic venules or terminal portal venules.

Broadening the perspective, investigations into the pathological significance of hepatocyte heterogeneity began with studies that investigated the perivenous damage resulting from hypoxia and drug toxicity.[147,148] Such studies shed new light on the well-documented observation that centrilobular hepatocytes are a common site of toxic damage by many hepatotoxins including acetaminophen, halothane, CCl_4 and bromobenzene. To elaborate on the dynamic changes in blood flow occurring in the hepatic artery and portal vein, a study performed in 1911 determined that the two circulations share a reciprocal interrelationship such that arterial flow compensates for reductions in venous flow, or alternately, an increase in arterial flow will result in a lowering of venous flow.[149] In either scenario, evidence that changes in hepatic arterial flow can alter the rate of hepatic drug metabolism raises questions surrounding its involvement in the many liver pathologies characterized by changes in portal venous blood flow.[150] This finding may be explained by the fact that only the hepatic artery perfuses the peribiliary plexus and biliary ductules, thus favoring a slightly greater exchange of arterial blood (compared to portal venous blood) with the biliary tract.[151] It is important to recognize that the large majority of studies conducted to date that have dealt with hepatocyte heterogeneity have involved the use of animal models. Much less is known regarding the zonal distributions of enzymes in human liver.

Since liver performs over 500 different functions, its metabolic demands are very high in comparison to all other organs. Bile synthesis is essential for proper nutrient and waste exchange, and thus represents one of the liver's main functions. Each day hepatocytes synthesize approximately 700 ml of bile; a complex mixture of cholesterol, bile salts, conjugated bilirubin, drug metabolites and various other metabolites. Hepatocytes govern haemoglobin turnover by producing and excreting conjugated bilirubin, a water-soluble complex consisting of concentrated haem protein. Protein metabolism is another major liver function. Catabolic processes include deamination of amino acids and conversion of the ammonia by-product to urea for renal excretion. Anabolic processes result in the production of many important plasma proteins including blood coagulation factors, complement proteins, haptoglobulin, C-reactive protein, transferrin serum enzymes and caeruloplasmin. Moreover, albumin is the principal protein synthesized by the liver. An important transport protein for many molecules, albumin is also an antioxidant and key regulator of blood oncotic pressure. Carbohydrate metabolism in the liver is an important process involving the dynamic incorporation of simple sugars into dense storage granules made up of glycogen. The liver releases vast amounts of heat during its robust metabolism of fats. Produced if necessary from carbohydrates or proteins, fats are stored or released into circulation as lipoproteins composed of cholesterol and phospholipids. Detoxification of drugs and most other potentially noxious substances involves their conversion to water-soluble compounds for excretion in bile or urine. Apart from their immune function, Kupffer cells aid hepatocytes with the delicate clearance of a number of serum glycoproteins by the process of endocytosis.[152]

LIVER PATHOLOGY

The consequences of liver damage have a great impact on body homeostasis. Despite being unique among the body's vital organs in that it can regenerate injured or diseased cells, repeated damage suffered by the liver over a prolonged period of time leads to irreversible impairment of function. Liver disorders often lead to systemic complications. Some of the more frequent changes and/or symptoms accompanying most forms of liver disease are listed below. Impaired bile synthesis results in poor absorption of dietary fats, lipid-soluble vitamins and calcium; symptoms of malnourishment as well as bone and muscle wasting appear. Coagulopathy results as levels of circulating coagulation proteins fail to be cleared or maintained. Circulatory changes may lead to portal hypertension due to elevated intrahepatic resistance; oesophageal varices are a telltale sign. The combination of increased portal pressure and decreased plasma oncotic pressure underlies the development of ascites. Anaemia and cyanosis contribute to changes affecting all parts of the body. Impaired renal circulation may evolve into hepato-renal syndrome, a condition punctuated by CNS toxicity and coma. In terms of immune function, splenomegaly leads to leucopoenia, and intrahepatic portal-systemic shunting seen in chronic liver disease drastically impairs Kupffer cell activity giving way to intestinally derived infections. In endstage cirrhosis, septicaemia or spontaneous bacterial peritonitis are common and often terminal events.

In the context of this investigation, liver diseases have been arbitrarily classified into the following five major etiologic categories: 1) Infectious: e.g. viral hepatitis; 2) Hepatocellular Disease: alcoholic cirrhosis; 3) Cholestatic: e.g. primary biliary cirrhosis; 4) Inherited and Metabolic Disorders: e.g. α_1-antitrypsin deficiency; and 5) Fulminant Hepatic Failure. The expression of arginase, NOS and MMPs in diseased livers was compared to that found in

surgical samples of apparently healthy human liver obtained during partial liver resection of isolated tumors. For each of the diseases or disease categories addressed in this study, some specific disease entities are briefly described below.

Hepatocellular Carcinoma is a highly malignant cancer that accounts for over 90% of all primary liver tumors. Morphologically, hepatocellular carcinoma can exhibit a gross pattern of unifocal, multifocal or diffusely infiltrative expression. The highest incidence of this neoplasm is often reported in individuals with cirrhosis secondary to chronic hepatitis B, though the chronic regenerative activity seen in other liver diseases (e.g. haemochromatosis, α_1-antitrypsin deficiency) is generally thought of as a potential precursor.[153] In unifocal disease, partial hepatic resection is a common course of therapy.

Metastatic Colon Carcinoma is not a liver disease itself however it is one of the most commonly recognized tumors to promote isolated hepatic metastases. For this reason, diagnosis or follow-up of this carcinoma often reveals the presence of the liver tumor. When present, hepatic metastases may be treated by a number of different approaches depending on the location and extent of the disease. Similar to hepatocellular carcinoma, cases of unifocal disease are commonly treated by partial hepatic resection.

Viral Hepatitis is the most common form of hepatitis. Contrary to popular belief, the widespread hepatic inflammation that characterizes this disease is not directly due to the virus itself but rather the vicious attack launched by infiltrating immune cells. Early diagnosis and supportive care may promote liver regeneration though the severity of the tissue destruction may call for orthotopic liver transplant.

Alcoholic liver disease is a composite of disease states evolving from the chronic consumption of alcohol. Initially, interference with the mobilization of triglycerides leads to a condition of hepatic steatosis (fatty liver). At some point an inflammatory reaction ensues that may gradually develop into chronic hepatitis, a condition marked by extensive scarring and destruction of liver cells. Chronic scarring in the liver underlies the development of cirrhosis, an irreversible condition that may eventually disrupt liver function enough to necessitate orthotopic liver transplant.

Toxic and drug-induced hepatitis occurs by either a direct or idiosyncratic means (e.g. by oral contraceptives). Destruction of liver tissue occurs very soon after exposure to the specific agent, the nature and quantity of which will determine the extent of injury. Serious damage requires OLT.

Primary Biliary Cirrhosis (PBC) involves the progressive inflammatory destruction of intrahepatic bile ducts. Possibly autoimmune in origin, females 40-59 years of age account for 90% morbidity. Progressing invariably to a cirrhotic liver, PBC is one of the best indications for OLT.

Primary Sclerosing Cholangitis (PSC) is a disease of unknown origin in which chronic fibrosing and inflammation of the entire biliary tract terminates in biliary cirrhosis. Nearly 2/3 of those affected are males. Therapy often demands OLT when patency of major bile ducts cannot be corrected by other means.

Alpha-1-Antitrypsin Deficiency is a metabolic disease in which the production of normal α_1-antitrypsin protein gives way to excessive trypsin activity that may result in liver failure and

pulmonary emphysema. OLT is particularly indicated in cases of severely dysfunctional α_1-protein abnormalities.

Biliary Atresia is a developmental anomaly encountered in children that is characterized by the absence or progressive obliteration of extrahepatic bile ducts. Treatment, when possible, consists of a substitution of the affected duct with a segment of jejunum, otherwise, OLT is the only course.

Cryptogenic Cirrhosis, as the name suggests is a cirrhotic liver disease of unknown origin. Because there is no sign of ongoing inflammation, its development has been suggested to occur secondary to a previously undetected inflammatory insult. Therapy requires OLT.

In *Budd Chiari Syndrome*, obstruction of the efferent hepatic vein is a rare condition of unknown aetiology affecting men and women equally, commonly between 20-39 years of age. In some instances a portacaval shunt may resolve the problem, though extensive blockage often necessitates OLT.

Autoimmune Hepatitis is a chronic, active inflammatory disease resulting in scarring and ultimately cirrhosis of the liver. Over 95% of the cases affect females, for whom early immunosuppressive therapy represents the only hope of avoiding the need for OLT.

Haemochromatosis (HC) is a liver disease in which the normal adult iron pool of 2-6 grams is increased to as much as 50-60 grams. Most commonly occurring in men, the cause of primary HC is hereditary, due to the presence of a gene that is closely linked to the human-leukocyte associated (HLA) locus. The excess iron eventually reaches toxic levels in the liver resulting in

micronodular pigment cirrhosis. Progressive destruction of the liver ultimately necessitates OLT.

Hepatic Epithelioid Hemangioendothelioma (HEHE) is a rare vascular tumor of the liver often affecting adult women which may consist of localized lesions but most often is a multifocal disease. Though radical resection may sometimes resolve the more localized form of HEHE, orthotopic liver transplant is a primary course of therapy for those with diffuse hepatic foci and severe liver dysfunction.

Fulminant Hepatic Failure (FHF) is a state of hepatic insufficiency involving massive (2-3 weeks development) to submassive (extending up to 3 months) hepatic necrosis. While FHF is quite uncommon, it is mainly caused by a rampant or fulminant form of viral hepatitis or drug (or chemical) toxicity. Beyond the immediate threat posed during the period of acute injury, the liver's remarkable capacity for regeneration can often lead to a complete recovery from fulminant hepatitis. Nevertheless, in those patients whose condition continues to deteriorate, OLT is the only remaining course of therapy.

Pathogenetically, *liver cirrhosis* represents the final common pathway of chronic liver injury. The ravaging effects of chronic liver disease are linked to fibrosis and distortion of intrahepatic microvasculature, fixation of intrahepatic resistance and a resultant loss in acinar architecture and normal liver compliance. The transition to a cirrhotic state follows any form of prolonged, repeated injury of parenchymal tissue and is characterized by the presence of isolated regenerative nodules. Pre- and post-hepatic portal hypertension markedly differ from intrahepatic portal hypertension in which irreversible fibrosis contributing to anatomical resistance coexists with a reversible increase in vascular tone.[154] Efforts to explain the

reversible component of increased vascular resistance implicate hepatic stellate cells,[155] decreased levels of hepatic endothelial vasodilators (e.g. NO, PGI_2, hyperpolarizing factor) or increased levels of vasoconstrictors (e.g. endothelin, prostanoids).[156,157] (for review, see Schiff ER et al.)[158]

L-Arginine-Metabolizing Enzymes: NOS and Arginase and Liver Function

L-Arginine is a semi-essential amino acid whose bioavailability depends primarily on a moderate level of protein consumption by healthy individuals. Once absorbed by the digestive tract, the majority of alimentary arginine is extracted from the portal system by the liver. For this reason and the fact that the liver is rich in various L-Arginine-dependent enzymes, hepatic metabolism of L-Arginine is greater than that found in any other region of the body. In contrast to D-Arginine, which has no bioactivity, L-Arginine is a substrate for five distinct enzymatic pathways, thus emphasizing its importance for human cell biology. These pathways include arginine decarboxylase, kyotorphine synthase, arginine-glycine transaminase, arginase and nitric oxide synthase.[159] Because of their importance to hepatic physiology, arginase and nitric oxide synthase shall now be discussed in much greater detail.

Nitric Oxide and Hepatic Physiology

In healthy liver, sinusoidal endothelial cells and hepatocytes are the main cell types known to express the NOS enzyme. Of the three known isoforms, endothelial NOS (eNOS) accounts for the major source of vascular NO. The mechanisms responsible for the regulation of transcription and translation of eNOS protein are not clear. Once the enzyme has been synthesized, its activity depends on two independent stimuli: intracellular calcium levels and the mechanical forces of shear stress acting at the cell surface. It is currently believed that

NO generated by endothelial cells acts as a key regulator of hepatic circulation.[160] Given the absence of smooth muscle in hepatic sinusoidal vessels, it has been postulated that the NO released by sinusoidal endothelial cells dilates the sinusoidal lumen by targeting perisinusoidal Ito cells. Nitric oxide regulates hepatic arterial and portal venous vascular tone, and these effects contribute to the modulation of hepatic metabolism.[161,162] Modulation of hepatic metabolism also results from the direct effects of NO on hepatic uptake, storage, detoxification and clearance mechanisms.[163] Considering the important physiologic actions of eNOS, any factors contributing to changes in the activity or expression of this enzyme are of great interest when studying liver diseases.

In the peripheral nervous system, neuronal NOS (nNOS) is believed to be an important source of the NO that modulates gastrointestinal smooth muscle cell contractility following its release from nonadrenergic, noncholinergic (NANC) pathways.[164] In various animal models, the functional significance of nNOS distribution in rat, trout and cat liver has been explored.[165,166,167] In each case, a rich plexus of nNOS-immunoreactive nerve fibers was found in the extrahepatic vessels (artery and portal vein) and ducts (biliary) that form the hepatic hilum. These findings suggest that the NO derived from these neurons may act as a neuromodulator of hepatic blood flow and hepatobiliary activity. Despite its well-established role in the human gastrointestinal tract, evidence of neuronal NOS (nNOS) activity in human liver has only been presented for gall bladder muscle[168] where it is thought to affect gall bladder motility.[169,170]

It is widely accepted that iNOS is expressed in many different cell types in response to inflammatory or immune stimuli. Interestingly, Armato and colleagues reported that

spontaneous induction of iNOS gene expression may occur in both neonatal and adult rat hepatocytes in culture.[171] The significance of this observation is as yet unclear.

Arginase and Hepatic Physiology

Within the last several years, arginase has drawn considerable interest as a substrate, having been suggested to act as a possible modulator of NOS activity. To date five different isoforms of this tetrameric metalloenzyme have been described; type I arginase is most abundant in the liver whereas the other four isoforms are variably distributed in other tissues such as erythrocytes, leukocytes, kidney and/or brain.[172] In contrast to its hepatic isoform, extrahepatic arginase serves only to regulate the intracellular levels of arginine and ornithine.

In the liver of ureotelic (urea producing) animals, arginase is the terminal enzyme of the hepatic urea cycle responsible for removal of the nitrogenous waste of protein catabolism (Illustration II-5). Compared to the glutamine synthetase pathway of ammonia removal, 95% of the ammonia load is typically removed via urea synthesis.[146] Its actions involve the irreversible hydrolysis of arginine to urea, a soluble waste product of ammonia, and ornithine, a precursor of proline and polyamines. Because proline and polyamines are respectively associated with collagen synthesis and cell division and differentiation, tight regulation of all isoforms of arginase throughout the body is essential in health. Quite unlike most other enzymes, mammalian arginase is activated by the reversible binding of manganese. Under physiologic conditions, variations in arginase expression coincide with similar changes in the other four urea cycle enzymes. In has been suggested however, that substrate availability (i.e. dietary protein) is a greater determinant of urea cycle activity than are pre-existing enzyme levels.[173]

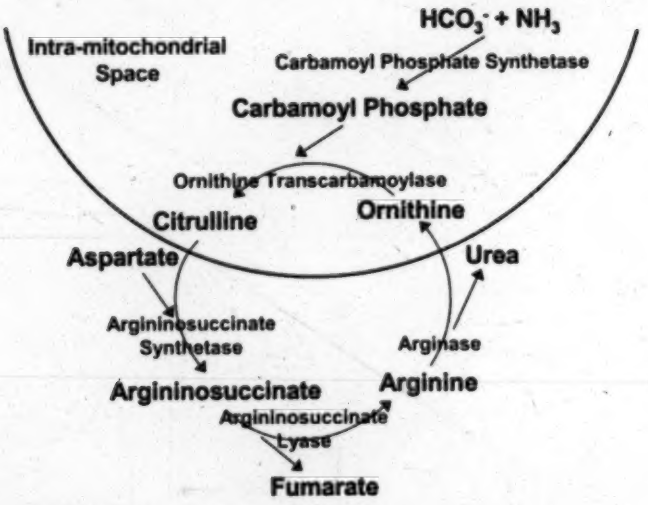


Illustration II-5: **THE HEPATIC UREA CYCLE** The five enzymes of the hepatic urea cycle are shown: two in the mitochondria and three in the cytoplasm. The cycle begins with the accumulation of ammonia (NH_3) in the mitochondria, which, along with bicarbonate (HCO_3) is converted to carbamoyl phosphate by carbamoyl phosphate synthetase. Ornithine availability in the mitochondria is important for the second step of the cycle which combines ornithine and CP to form citrulline. The three remaining reactions occur in the cytosol, to where citrulline translocates and combines with aspartate to form argininosuccinate. Next argininosuccinate lyase hydrolyzes argininosuccinate to fumarate and arginine, the latter being converted by the final urea cycle enzyme, arginase, to urea and ornithine.

Outside of its predominant role in the hepatic urea cycle, arginase has been linked to some forms of immune response. Studies show that increased synthesis and release of arginase from macrophages leads to depletion of arginine in the microenvironment, thus resulting in anti-tumour, anti-parasitic, and anti-viral defence.[174] During an immune response, the release of known inducers of arginase like PGE_2 or Th2 cytokines IL-4, IL-10[175] and TGF-β[176] may be involved in its induction.

Nitric Oxide and Hepatic Pathology

As a major organ of digestion, the liver is exposed to the first passage of nutrients absorbed by the digestive tract. Consequently, this renders the liver highly susceptible to absorbed pathogens, toxins etc. Moreover, its function as a division of the reticuloendothelial system

(RES) makes it quite susceptible to becoming an early site of immune response to invading microorganisms. Indeed the hepatic RES contains a unique population of resident macrophages (Kupffer cells) that play an important role in the immune reactions of this organ.

A number of recent investigations into the pathogenesis of liver disease have provided sound evidence that, in addition to Kupffer cells, hepatocytes may contribute to the overall immune response of the liver. Strategically located in the hepatic sinusoid, Kupffer cells are readily exposed and especially sensitive to virtually all forms of noxious stimuli that may pass through the liver. Exposure to LPS for example is a reliable stimulus for the activation of human Kupffer cells.[177] Experiments performed *in vivo* have confirmed that the ratio of Kupffer cells to hepatocytes is increased following injection of *Mycobacterium bovis* or Bacillus Calmette-Guérin (BCG) ± endotoxin.[178] Once activated, Kupffer cells can synthesize an array of pro-inflammatory mediators including superoxide, NO, eicosanoids, TNF and IL-1.[163] Accordingly it has been found that plasma levels of TNFα are increased in patients suffering from liver failure.[179] Moreover, the diffusion of TNF to nearby hepatocytes leads to their recruitment as a newly derived source of iNOS.[180] Though controversy still surrounds the nature of the stimulus for hepatocyte iNOS activation, the relative abundance of these cells combined with their demonstrated sensitivity to Kupffer cell-derived proinflammatory cytokines supports the current theory that hepatocytes, rather than Kupffer cells are the major source of NO produced in inflamed liver.[181]

A sudden burst in intrahepatic iNOS can lead to the generation of supraphysiologic levels of NO. Because of its calcium independence, iNOS is generally thought to maintain its activity for as long as its structure remains intact. In the liver, however, this theory is challenged by the

substrate competition that could conceivably exist between NOS and arginase (see pg. 71: Interactions Between NOS and Arginase). When produced at high concentrations, NO itself becomes cytotoxic partly through its ability to inactivate cytochrome C oxidase, aconitase and ribonucleotide reductase. As for other important oxidants, superoxide is also produced in high concentrations during liver failure.[182] When large amounts of NO are generated in regions that superoxide is co-produced, the two molecules spontaneously react at a near diffusion rate to form peroxynitrite ($ONOO^-$). Peroxynitrite is a lethal oxidant that targets functional groups of various enzymes, or it may react further, with a proton to produce a hydroxyl radical. In contrast, NO may be re-released following the metabolism of peroxynitrite to S-nitrosothiols, and this pathway is believed to account for peroxynitrite-dependent inhibition of vascular reactivity.[183] Therefore, the role of peroxynitrite in the development of a clinical syndrome compatible with vascular hyporeactivity in liver transplant patients is a matter of great interest.

In inflammatory liver disease, much of the evidence for the involvement of NOS enzymes has come from the work of TR Billiar and colleagues using various animal models.[184] The amounts of NO generated are the key determinant of whether anti-inflammatory or cytotoxic effects prevail. For example, by measuring the extent of microvascular leakage of endotoxin-induced shock it has been demonstrated that basal NO has a protective effect whereas high amounts of NO generated by iNOS contribute to vascular endothelial damage.[185] Interestingly, the addition of monophosphoryl lipid A to human monocytes has produced an up-regulation of particulate NOS (eNOS) activity.[186] Likewise, it is believed that basal amounts of NO generated by hepatocytes may be modified by liver disease. Indeed, Rockey and Chung[187] found a reduction in hepatic eNOS activity without any change in eNOS mRNA levels in cirrhotic rat liver. Their data show not only that NO production by hepatic eNOS is

levels in cirrhotic rat liver. Their data show not only that NO production by hepatic eNOS is altered in liver disease, but also that the mechanisms responsible for this alteration may involve post-translational modification of the enzyme rendering eNOS less active.

An extensive amount of work has been published on the topic of iNOS and liver disease. Most of these studies focused on the use of cultured cells and models of liver disease. In terms of cellular distribution, a number of studies have shown that Kupffer cells, Ito cells, hepatocytes and even endothelial cells are all potential sources of iNOS.[188,189,181] Furthermore, it has been shown that iNOS expression in liver varies temporally, spatially, and even with the type of liver disease.[190] Indeed, in response to endotoxin or proinflammatory cytokines such as TNFα, IL-1 and IFN-γ as well as their combinations, iNOS is upregulated within hours in hepatocytes and Kupffer cells. Moreover, these stimuli can act synergistically to induce iNOS expression.[191] Interestingly, superoxide has been shown to induce IL-1β-dependent iNOS upregulation in rat hepatocytes.[192]

In contrast to endotoxin and cytokines, some growth factors and glucocorticoids down-regulate the expression of iNOS. A summary of factors that control hepatic iNOS expression is given in Table II-1.[193]

TABLE II-1

EFFECTORS OF HEPATIC iNOS EXPRESSION

FACTOR	EFFECT ON EXPRESSION
Lipopolysaccharide	+
Pro-Inflammatory Cytokines (TNFα, IL-1β, IFN-γ)	+
Prostaglandins	+
Phorbol Esters	+
Nitric Oxide	-
p53	-
Dexamethasone	-
Hepatocyte Growth Factor	-
Epidermal Growth Factor	-
TGF-β	-

NOTE: TNFα = tumor necrosis factor-α, IL-1β = interleukin-1β, IFN-γ = interferon-γ, p53 = 53 kDa tumor suppressor protein, TGF-β = transforming growth factor-β, + = stimulatory, - = inhibitory

A current review of the literature indicates that the expression of iNOS may have both cytoprotective and detrimental effects in the liver. These may be dependent upon the nature of pathogenetic stimuli (e.g. chemical or bacterial toxins, hemorrhagic shock and ischemia-reperfusion injury), as well as on non-specific (L-NAME, L-NMMA) or specific (1400W)[194] inhibitors of iNOS that are used to probe the significance of iNOS expression for liver function. A synopsis of effects of inducible NO under various experimental conditions is given in Table II-2.[193]

TABLE II-2

PROTECTIVE AND DETRIMENTAL EFFECTS OF iNOS INDUCTION DURING LIVER DAMAGE

STIMULUS	NET EFFECT OF NO	MECHANISM OF NO EFFECTS
TNFα, Fas antibody	Protective	Inhibits caspases and apoptosis Induces HSP-70
H_2O_2	Protective	Induces heme oxygenase-1
Acetaminophen	Protective	Modulates reduced glutathione levels
Endotoxemia	Protective	Inhibits apoptosis
Endotoxemia	Toxic	Oxidative stress and circulatory failure
TNFα + *N*-galactosamine	Protective	Inhibits apoptosis
Carbon tetrachloride	Protective	Reduces oxidative stress
Liver regeneration	Protective	Inhibits apoptosis
Ischaemia-reperfusion	Toxic	Oxidative damage
Hemorrhagic shock	Toxic	Direct toxicity, proinflammatory
Alcoholic liver injury	Protective	Not determined

NOTE: TNFα = tumor necrosis factor-α, H_2O_2 = hydrogen peroxide, HSP-70 = 70 kDa heat shock protein. The upper three cells (shaded) correspond to studies conducted *in vivo*; all others below were performed *in vitro*.

Arginase and Hepatic Pathology

Absence of the hepatic isoform of arginase occurs in hyperargininemia, a rare autosomal recessive condition hallmarked by elevated levels of circulating arginine and subsequent neurologic abnormalities and growth retardation.[195] Aside from hereditary deficiencies however, changes in arginase activity have mainly been associated with fibrosis and cirrhosis of the liver.

In 1965 it was first reported that the level of arginase activity in human cirrhotic liver was 50% below that found in normal liver.[196] A 1974 study examined the specific activity of arginase in alcohol-induced cirrhotic human liver (obtained during implantation of a portal-systemic shunt) and found that it was reduced by about 69%.[197] This reduction was believed to be due

to diminished protein synthesis. In another study, arginase activity in liver biopsy specimens dropped by about 50% in both cirrhotic & chronic-active hepatitis diseased liver, though there was no change in chronic-persistent hepatitis.[198]

Interactions Between NOS and Arginase

Despite its benefits to immune function, the depletion of L-arginine by arginase is thought to be a detriment to NOS activity. Accordingly it is remarkable to find that N^G-hydroxy-L-arginine, an intermediate of NO synthesis, has been determined to be a potent inhibitor of arginase.[199] In fact, its $K_i = 42\ \mu M$ for rat liver arginase is even lower than the K_M (1-1.7 mM) of arginase for L-arginine.[64] These observations have prompted a number of studies that have since described a pattern of reciprocal regulation occurring between the arginase and NOS, predominantly in cases of macrophage-mediated non-specific immunity.[199,200,201,202] For example, several days after injury arginase activity in wounds and other inflammatory sites began to rise as macrophage-derived NO levels began to decline, i.e. an early inflammatory response was followed by a later phase of repair. One explanation for this observation may be the transition from a Th1 cytokine profile to Th2, since Th2 cytokines TGF-β, IL-4 and IL-10 are known suppressors of NO synthesis.[175,176]

Indeed the nature of the stimuli that evoke changes in arginase or NOS activity is a matter of key importance. In rat aortic endothelial cells, LPS was shown to induce both iNOS and arginase whereas addition of cytokines resulted in high output production of N^G-hydroxy-L-arginine (and NO) by iNOS. Under these conditions a profound inhibition of arginase activity was detected.[203]

In terms of L-arginine affinities, the published K_M for human hepatic arginase is 4.7 mM[203] compared to 1-10 μM[204] for eNOS. Despite this difference, little is known about the level of interaction between arginase and NOS in human liver disease.

MMPs and Liver Function

MMPs and Hepatic Physiology

Connective tissue and the extracellular matrix combine to provide vital scaffolding for highly parenchymal organs such as the liver. In healthy liver, the extracellular matrix of the hepatic sinusoids consists mainly of collagens III and IV whereas collagen type I is the main collagen found in portal tracts and the walls of hepatic veins. The physical and chemical stresses that are normally incurred by connective tissue make it a highly active site of cell matrix synthesis and degradation. The importance of maintaining a delicate balance to this process is evident at both the tissue level affecting growth, repair and regeneration, as well as the cellular level where it affects many aspects of cell adhesion, migration, differentiation and activity. To manage these tasks liver cells differentially express MMPs in addition to some of their natural inhibitors (TIMPs). MMP-2 and MMP-9, in particular, play an important role in the remodeling reactions of liver. It has been shown that early human liver development is associated with increased expression of MMP-2 in growing microvascular endothelium and hepatocytes.[205] Moreover, MMP-2 RNA was found in normal adult liver.[206] Finally, a change in the balance between TIMP-1 and MMP-2 may underlie the regulation of extracellular matrix synthesis during liver regeneration after partial hepatectomy in rats.[207] All of the data point to a crucial role for the MMP/TIMP system in the physiologic function of liver.

A delicate balance between the synthesis and degradation of the extracellular matrix is clearly disturbed in a variety of inflammatory liver disorders. Indeed, liver fibrosis,[208,209,210,211] as well as viral liver disease[212,213,214] has been linked to an imbalance in the MMP/TIMP system. Interestingly, of the many abnormalities characterizing chronic liver disease, an important event contributing to the development of fibrosis in the hepatic architecture involves activation of normally resting Ito cells.[215] Dispersed throughout the space of Dissë, these cells respond to liver damage by migrating to zone 3 of the liver acinus where they adopt a contractile myofibroblastic phenotype that secrete collagen types I, III and IV, and laminin. As a result, sinusoidal blood flow is disturbed and at the same time hepatocytes experience reduced access to protein-bound nutrients.[216,217] Aside from enhancing collagen deposition, activated Ito cells also alter extracellular matrix degradation by secreting a cocktail of MMPs (including MMP-2) as well as some of their natural inhibitors (TIMPs).[218] It is thought that a net accumulation of matrix proteins (primarily collagen types I and III) during chronic liver disease is a result of the reciprocal actions of TNFα and TGF-β on the expression of these two proteins.[219]

Several groups have focused on characterizing the patterns of expression of MMPs and their inhibitors (TIMPs) in various liver diseases. Animal studies have determined a number of important characteristics, such as the finding that MMP-9 appears to be predominantly produced by Kuppfer cells, and MMP-2 by Ito cells.[219] Furthermore, the expression of MMP-2 by activated Ito cells is linked to the co-expression of MT-MMP and TIMP-2.[219,220] Together these proteins coordinate the activation and release of active MMP-2 that in turn seems to influence the proliferation of Ito cells in an autocrine pathway. Indeed the developing role of

MMP-2 in liver disease has prompted one group to investigate the expression of the various isoforms of MT-MMPs associated with its activation. It was found that MT2-MMP expression mainly occurs in hepatocytes and biliary epithelial cells while stromal cells and activated Ito cells produce high levels of MT1-MMP.[221] Similar results were reported by another group who localized MT1-MMP and MMP-2 to Ito cells and fibroblasts.[222] This observation supports an earlier suggestion that hepatocytes-Ito cell interactions are important for the activation of Ito cell-derived MMP-2.[223]

Some studies have been based on the hypothesis that the release of MMPs into blood circulation is an index of local MMP activity occurring at a site of tissue inflammation.[224] In patients with chronic hepatitis C, the expression of MMP-2 and MMP-9 in blood, leukocytes and liver was compared. The results showed a differential pattern of mRNA expression: MMP-2 was almost exclusively localized to the liver whereas MMP-9 was predominantly expressed in leukocytes.[225] The ratio of circulating MMP-2:TIMP-1 was correlated with both fibrosis and inflammatory activity in liver biopsies. Interestingly, the levels of circulating MMP-2 and MMP-9 did not correlate with those found in peripheral blood leukocytes. Currently, little is known about circulating MMPs in human liver disease. Another study compared hepatic levels of MMPs and TIMPs to those found in the blood of rats suffering from biliary fibrosis. The activities of MMP-2 and MMP-9 in liver were significantly elevated by day-2 following bile duct ligation, and remained high throughout the 30-day study period.[226] Likewise, TIMP levels in diseased liver were consistently high. In contrast, there were no reported changes in the activities of these enzymes in plasma. Serum levels of TIMP-1 were measured in patients with genetic haemochromatosis and were found to correlate to the concentration of hepatic iron levels whereas serum MMP-2 correlated to the degree of

histological fibrosis.[227] The ratio of MMP-2:TIMP-1 was significantly lower in patients than controls. Another study looked at a group of 98 patients with chronic hepatitis C to also determine the levels of MMP-2 and TIMP-1 in serum. TIMP-1 levels positively correlated with the degree of fibrosis, whereas MMP-2 best correlated to the degree of periportal necrosis. Non-responders to IFN-β therapy had markedly higher MMP-2 levels than those who responded transiently or for a sustained period. The ratio of MMP-2:TIMP-1 was significantly higher in patients not experiencing a sustained response to therapy.[228]

A growing number of perspectives of the role of MMPs and TIMPs in liver disease are appearing in the literature. The recurring theme appears to emphasize the role of hepatocytes and Ito cells in the generation and regulation of MMP-2 and TIMP-1. Evidence for the differential expression of these proteins by various cell types in diseased liver emphazises the need for their further characterization. Likewise, the reported correlations between these enzymes and indices of liver damage continue to make their measurement in blood an compelling approach to non-invasive diagnostics.

MATERIALS AND METHODS

SELECTION OF SUBJECTS

Blood and liver specimens were collected from 46 adult patients who received orthotopic liver transplantation for end-stage liver disease between the months of January 1997 to May 1998. Post-operative pathology reports were reviewed, confirming a distribution of major disease groups including viral hepatitis, alcoholic cirrhosis, cholestasis and fulminant hepatic failure (herein referred to as major liver disorders). In addition, uncommon forms of liver disease including haemochromatosis, cryptogenic cirrhosis, autoimmune hepatitis, α_1-anti-trypsin disorder, endothelioid hemangioendothelioma and Budd Chiari syndrome (referred to herein as rare liver disorders) were also diagnosed. Each patient provided written informed consent prior to his/her recruitment into the study.

Control liver sections were obtained from adult patients who received a partial hepatectomy for select intrahepatic malignancies (hepatocellular carcinoma or metastatic colon carcinoma). Only those patients diagnosed with localized tumors were considered. Appropriate control specimens were obtained from the outermost region of the tumor-free margin from the excised lobe.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and those of the Research Ethics Board of the University of Alberta, Faculty of Medicine.

SPECIMEN COLLECTION

All liver tissue samples including those obtained from patients undergoing liver transplant were

collected intraoperatively at the time of excision at which time they were immediately sectioned for storage in eppendorf tubes, snap frozen using liquid nitrogen, then stored until assayed at -80 °C.

All control group blood was obtained intravenously from healthy adult donors who had not taken any drugs for at least 2 weeks prior to collection. Blood from liver transplant patients was collected once prior to the pre-operative dose of azathioprine (venous); once post-operatively prior to the first dose of cyclosporine (arterial); and once daily thereafter for another four consecutive days (arterial or venous). At the time of collection of donor or patient blood, 9 ml of blood was added to 1 ml of 3.15% sodium citrate and immediately centrifuged at 800 x g for 10 min at room temperature. Plasma was then aliquoted and stored until assayed at -80 °C.

MEASUREMENT OF NITRIC OXIDE SYNTHASE AND ARGINASE ACTIVITIES

NO synthase and arginase activities were measured by the rate of conversion of U-^{14}C-L-arginine to U-^{14}C-L-citrulline[229] and ^{14}C-urea,[230] respectively. Briefly, liver samples (0.5 g wet weight) were homogenized by sonication (VibraCell, Danbury, CT) in 1 ml of ice-cold homogenization buffer (pH 7.4) containing 50 mM Tris-HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 10 mM $MnCl_2$ (arginase assay) followed by centrifugation at 10,000 x g for 20 min at 4 °C. Following centrifugation, 40 μl of supernatant was incubated at 37°C for 20 min in assay buffer (pH 7.4) containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM L-citrulline, 20 μM L-arginine, 1.5 mM dithiothreitol, 1.5 mM NADPH, 10 μM tetrahydrobiopterin, 10 μM F[illegible], 10 μM FMN and U-^{14}C-L-arginine (0.5 μCi/ml, Amersham, Oakville, ON). The specificity of L-arginine conversion by NOS to L-citrulline and by arginase to urea was further

confirmed using the respective inhibitors: 1.2 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME)[231] or 10 mM N^{G}-hydroxy-L-arginine (L-NOHA)[199,232,203] (Alexis Corporation, San Diego, CA). Additionally, 1.5 mM ethylene glycol-bis [β-aminoethyl ether] N,N,N'-tetraacetic acid (EGTA),[233] a calcium chelating agent, was used to differentiate between Ca^{2+}-dependent and -independent isoforms of NOS. All enzyme activities are expressed as pmol product generated per minute per mg protein.

WESTERN BLOT OF NOS ISOFORMS

The expression of NOS isoforms in liver sections was measured as described before.[233] Briefly, homogenized samples (80 μg protein each) were subjected to 7% SDS-PAGE under reducing conditions. Proteins were wet-transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH) using a Trans-Blot Cell system (Bio-Rad, Mississauga, ON). The eNOS and nNOS were identified using respective rabbit anti-human polyclonal antibodies (Santa Cruz, La Jolla, CA, 0.2 μg ml^{-1}) whereas mouse anti-human monoclonal antibodies (Transduction Laboratories, Lexington, KY, 0.05 μg ml^{-1}) were used for iNOS. All blots were developed simultaneously using an ECL kit (Amersham Canada Ltd, Oakville, ON) and the density of bands was quantified using a ScanJet 3c scanner (Hewlett Packard, Boise, ID) and SigmaGel measurement software (Jandel Corporation, San Rafael, CA). Band densities are expressed in arbitrary units.

IMMUNOHISTOCHEMISTRY OF NOS ISOFORMS

Immunohistochemistry was carried out using an antigen retrieval technique and specific anti-eNOS and anti-iNOS monoclonal antibodies. Samples were prepared by mounting 4 μm thick slices of formalin-fixed/paraffin-embedded liver sections onto Aptex coated slides and

drying them overnight at 37°C. Slides were then heated for 10 min at 70°C and immediately immersed into xylene for deparaffinization followed by rehydration with decreasing grades of ethanol, and finally with water. Endogenous peroxide was quenched with an 8% H_2O_2/60% methanol solution for 6 min and later rinsed with water. Antigen retrieval was accomplished by microwaving slides in citrate buffer. Counterstaining was done with Harris' haematoxylin. Blocking reagent (20% normal goat serum) was applied to each slide followed by 15 min incubation at room temperature. Mouse anti-human monoclonal antibodies for eNOS and iNOS (Transduction Laboratories, Lexington, KY, 5 $\mu g\ ml^{-1}$) were used. Alternately, mouse IgG was used for negative controls. Following 30 min incubation with the primary antibody, slides were rinsed with phosphate-buffered saline (PBS) for 5 min and then incubated for an additional 20 min with the link reagent (VectorLab, Burlingame, CA; biotinylated anti-mouse IgG [2-10 $\mu g\ ml^{-1}$]). After a 5 min PBS rinse, the streptavidin peroxidase label reagent (Biogenex, San Ramon, CA) was applied for 20 min at room temp. Next, a final PBS rinse was followed by 5 min incubation with a chromagen solution containing DAB (3,3'-diaminobenzidine) and H_2O_2. Last, the slides were dehydrated, cleared and mounted for viewing.

SERUM LIVER ENZYMES

Serum levels of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were obtained from patient medical records following their measurement by University of Alberta Hospital laboratory personnel using an Hitachi 917 multi-channel automated analyzer. Photometric assays of the following reactions were carried out to determine the level (IU/l) of each enzyme. In a solution containing α-oxoglutarate and L-aspartate, aspartate aminotransferase catalyzes oxaloacetate generation that is reduced to L-malate by NADH,

lactate dehydrogenase and malate dehydrogenase. The process of deamination of L-alanine by alanine aminotransferase generates pyruvate that is reduced by NADH to lactate in the presence of lactate dehydrogenase. Alkaline phosphatase generates p-nitrophenol in its reaction with p-nitrophenyl phosphate. Limits of detection for each enzyme were 5 IU/l, 5 IU/l and 2 IU/l, respectively.

PROTEIN ASSAYS

The protein content of liver homogenates and plasma was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Dilution factors for tissue samples were determined empirically by ensuring that their resultant spectral reading was well within the range of those determined for the protein standard series: typically 1:75 liver homogenates; 1:100 plasma. Plasma samples were diluted with normal saline and liver homogenates were diluted with homogenizing buffer. Preparation of protein standards was completed using lyophilized bovine serum albumin (Bio-Rad Laboratories, Hercules, CA) in the same diluent, arriving at a series of concentrations ranging from 0-1000 µg/ml. Once samples and standards were diluted, 200 µl of Bio-Rad assay reagent (diluted earlier with distilled water 1:5) was added to each well on a 96-well plate (Corning Glass Works, Corning, NY). Next, 10 µl of sample or standard was added to each well immediately after which the plate was placed into a microplate reader (Bio-Rad Laboratories, Hercules, CA) to measure absorbance at the 595 nm wavelength.

PLASMA NITRITE AND NITRATE LEVELS

Cell-free plasma was deproteinized by ultrafiltration using an Ultrafree-MC micropartition system (Millipore Corporation, Bedford, MA). Samples were analyzed using an automated

HPLC system according to the method of Green et al.[234] The method of detection was based on the nitrite reaction with Griess reagent to give a colored product that could be detected at the visible wavelength of 546 nm. The nitrate content of the plasma sample was reduced to nitrite as it passed through a cadmium precolumn before being mixed with the Griess reagent and then analyzed on-line using a visible light absorbance detector. To account for abnormalities in renal function the resulting levels were also normalized to serum creatinine levels that were determined as described below. The limit of detection is 1.0 nmol nitrite/ml.

LIVER MMP-2 AND MMP-9 LEVELS

The activities of gelatinase enzymes MMP-2 and MMP-9 were measured by zymography[235] of supernatants from samples of homogenized liver tissue prepared as described above (see pg. 77: Measurement of Nitric Oxide Synthase & Arginase Activities).

Briefly, non-heated samples (containing 12 μg protein) were mixed with SDS sample buffer (without 2-mercaptoethanol) and applied to 8% polyacrylamide separating gels co-polymerized with 2 mg/ml of gelatine (Sigma-Aldrich Canada Ltd., Oakville, ON.). After electrophoresis, the gels were washed with 2% Triton X-100 to remove SDS, then left in incubation buffer (0.15 M NaCl, 5 mM $CaCl_2$, 0.05% NaN_3 and 50 mM Tris-HCl buffer, pH 7.5) at 37°C overnight to facilitate the development of gelatinase activities. After incubation the gels were stained with 0.05% Coomassie brilliant blue G-250 (Sigma, St. Louis, MO) in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and de-stained in a mixture of methanol: acetic acid: water (0.5: 1: 11). Gelatinolytic activities were detected as transparent bands against the surrounding Coomassie brilliant blue-stained gelatine. Prestained low range molecular weight standards (14-97 kDa; Bio-Rad, Mississauga, ON) were used for reference. Standards for pro-

and active-forms of MMP-9 and MMP-2 came from cell media produced from cultures of the human fibrosarcoma cell line HT-1080.

To quantify the activities of the detected enzymes, zymograms were digitally processed using a ScanJet 3c scanner (Hewlett Packard, Boise, ID). The intensities of the separate bands were analyzed using SigmaGel densitometry software (Jandel Corporation, San Rafael, CA). For reference, a representative photo of a zymography gel is shown below (Illustration II-6). In some experiments 1,10-phenanthroline (0.1 mM), a known inhibitor of MMPs, was added to the incubation buffer and following overnight incubation the gels were stained and analyzed as described above. As little as 1 pg of enzyme is detectable using this method.[236]

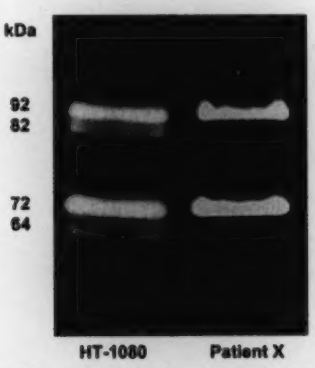


Illustration II-6: **REPRESENTATIVE ZYMOGRAPHY BLOT** Reference bands corresponding to proMMP-9 (92 kDa), MMP-9 (82 kDa), proMMP-2 (72 kDa) and MMP-2 (64 kDa) can be seen on the HT-1080 source lane for comparison with any given patient sample.

PLASMA MMP-2 AND MMP-9 LEVELS

Samples of plasma isolated from healthy donors and liver transplant patients were subjected to zymography (15 μg protein per well) using the same methods described above (see pg. 81).

SERUM CREATININE LEVELS

Serum levels of creatinine were obtained from patient medical records following their perioperative collection and measurement by University of Alberta Hospital laboratory personnel using an Hitachi 917 multi-channel automated analyzer. The method was based on a photometric assay of the rapid reaction between creatinine and picric acid. The limit of creatinine detection was 5 mM.

REAGENTS

Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM and were analyzed by Instat software (GraphPad Instat Software, Inc.). Comparisons were performed using either one-way analysis of variance followed by Tukey-Kramer multiple comparisons test or the Kruskal-Wallis nonparametric one way analysis of variance test followed by Dunn's multiple comparisons test. When appropriate, comparisons were made by either the two-tailed unpaired Student t-test or the non-parametric Mann-Whitney test. Differences were considered statistically significant at probability values < 0.05.

RESULTS

PATIENT DIVERSITY

Table II-3 summarizes patient demographics and some routine clinical and laboratory parameters. Among the 42 patients admitted into the study, 24 were female. The serum level of aspartate aminotransferase (AST) is typically increased 10-100 fold above the normal value (< 40IU/l) in cirrhotic liver, acute hepatitis, infectious mono and hepatic necrosis. Compared to AST, alanine aminotransferase (ALT) is a relatively more specific indicator of liver malfunction since it is primarily found in the liver where serum levels are variably increased with hepatocellular disease, active cirrhosis, biliary obstruction, and hepatitis. In the absence of bone disease or pregnancy, elevated levels of serum alkaline phosphatase (AP) may accompany parenchymal disorders such as hepatitis and cirrhosis but often reflect impaired biliary tract function. Diabetes mellitus is a metabolic disease resulting in reduced carbohydrate utilization and enhanced lipid and protein consumption. Because glucose homeostasis is often affected by liver disease, these abnormalities along with long-term complications such as neuropathy, retinopathy, nephropathy, vasculopathy and increased susceptibility to infection make diabetics with liver disease an especially challenging patient group to treat.

TABLE II-3

DEMOGRAPHICS AND CLINICAL AND LABORATORY PARAMETERS OF LIVER TRANSPLANT PATIENTS

DISORDER	N	FEMALES	AGE (YEARS)	AST	ALT	AP	DIABETES MELLITUS
VH	9	5	51± 3.3	466± 369	334± 261	199± 31	0
AC	7	3	51± 1.7	47± 4	14.5± 1	140± 19	1
PBC	8	7	55± 3.8	156± 43	130± 102	350± 74	1
PSC	3	3	34± 4.7	202± 103	184± 8	451± 292	0
BILIARY ATRESIA	4	3	1.4± 0.2	665± 636	297± 49	310± 194	0
FHF	4	1	43± 10	451± 211	375± 26	180± 37	0
HC	2	0	59	117	n/a	429	1
HEHE	1	0	37	20	n/a	90	0
CC	1	0	66	41	n/a	66	0
AI-HEP	1	1	25	114	93	248	0
α_1-ANTI-TRYPSIN	1	0	56	82	n/a	n/a	0
BUDD-CHIARI	1	1	54	68	n/a	173	1

NOTE: Age and liver enzyme values are expressed as mean or mean ± SEM wherever possible. AST = serum aspartate aminotransferase and normal clinical values are < 40 IU/l, ALT = serum alanine aminotransferase and normal clinical values are < 50 IU/l, AP = serum alkaline phosphatase and normal clinical values are 30-130 IU/l. VH = Viral Hepatitis, AC = Alcoholic cirrhosis, PBC = Primary biliary cirrhosis, PSC = Primary sclerosing cholangitis, FHF = Fulminant hepatic failure, HC = Hemochromatosis, HEHE = Hepatic epithelioid hemangioendothelioma, CC = Cryptogenic cirrhosis, AI-Hep = Autoimmune hepatitis, n/a = data not available

CONVERSION OF L-ARGININE BY ARGINASE AND NOS IN NORMAL VS. DISEASED LIVER

Hepatic arginase was clearly the dominant L-arginine metabolizing enzyme in both normal and diseased human liver. Relative to NOS, the proportion of L-arginine metabolized via the arginase pathway amounted to 94-96% of total activity. In normal liver, the rate of conversion of L-arginine to urea was 24.3 pmol/min/mg protein. This was not significantly changed by the end-stage liver diseases, as L-arginine was processed at an average rate of 22.0 pmol/min/mg protein under these conditions (Fig. II-1).

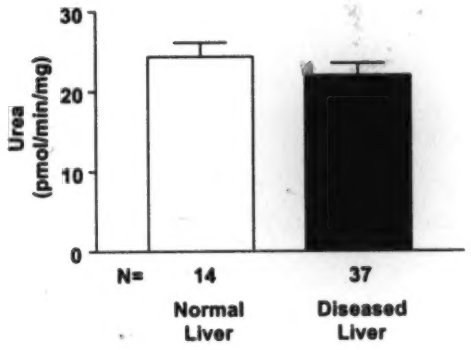


Figure II-1: **L-ARGININE CONVERSION VIA ARGINASE IN NORMAL VS. DISEASED LIVERS** The mean intrahepatic activity of arginase in normal control liver is compared with liver of various end-stage diseases. Activity is expressed as pmol urea production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM.

The remaining 4-6% of L-arginine conversion in normal and diseased liver was associated with the eNOS and iNOS enzymes. In both normal and diseased liver L-arginine turnover via eNOS was relatively constant and amounted to 0.39 vs. 0.33 pmol/min/mg protein, respectively (Fig. II-2).

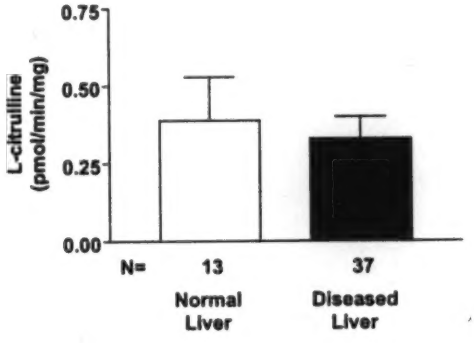


Figure II-2: **L-ARGININE CONVERSION VIA eNOS IN NORMAL VS. DISEASED LIVERS** The mean intrahepatic activity of eNOS in normal control liver is compared with liver of various end-stage diseases. Activity is expressed as pmol L-citrulline production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM.

In contrast to eNOS, there was a significant increase in the overall activity of iNOS in diseased vs. normal livers. Indeed, in normal livers iNOS accounted for a total of 2% of L-arginine conversion while in diseased livers this value was doubled (Fig. II-3).

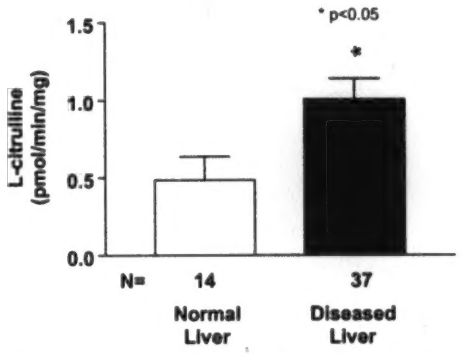


Figure II-3: **L-ARGININE CONVERSION VIA iNOS IN NORMAL VS. DISEASED LIVERS** The mean intrahepatic activity of iNOS in normal control liver is compared with liver of various end-stage diseases. Activity is expressed as pmol L-citrulline production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

ARGINASE ACTIVITY IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

Upon grouping data into subsets of major forms of liver disease it was apparent that intrahepatic arginase activity in viral hepatitis, alcoholic cirrhosis and cholestasis did not differ from the control. In contrast, arginase activity was significantly reduced in fulminant hepatic failure (Fig. II-4).

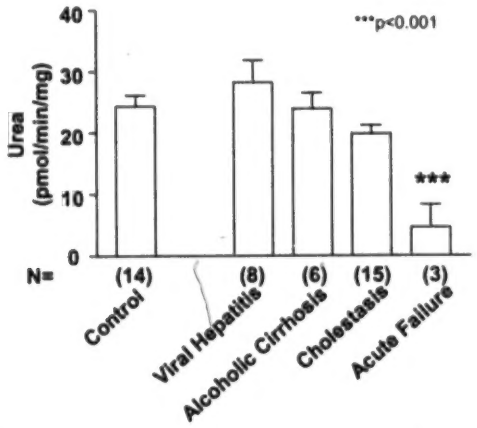


Figure II-4: **ARGINASE ACTIVITY IN NORMAL VS. MAJOR LIVER DISORDERS**
The mean intrahepatic activity of arginase in normal control liver is shown along with livers of various end-stage diseases. Activity is expressed as pmol urea production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

eNOS ACTIVITY IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

No significant difference in intrahepatic eNOS activity was found in any of the major liver disorders when compared to the control levels (Fig. II-5).

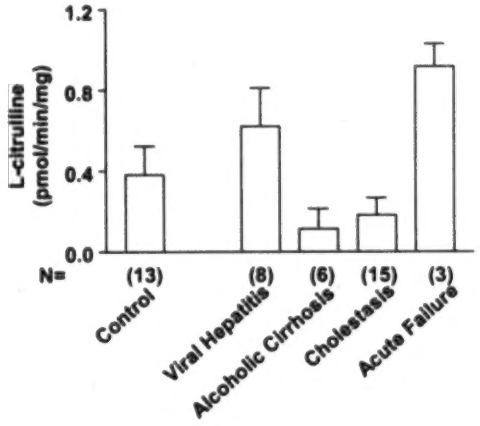


Figure II-5: **eNOS ACTIVITY IN NORMAL VS. MAJOR LIVER DISORDERS** The mean intrahepatic activity of eNOS in normal control liver is shown along with livers of various end-stage diseases. Activity is expressed as pmol L-citrulline production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM.

eNOS AND nNOS EXPRESSION IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

The Western blot analysis revealed that eNOS immunoreactivity was present in the liver samples of control patients. This was not significantly changed in samples from patients with alcoholic cirrhosis, cholestasis and acute liver failure. In contrast, the eNOS immunoreactivity was significantly reduced in the livers of patients with viral hepatitis (Fig. II-6).

The immunoreactivity of nNOS was not detected in normal or diseased liver samples.

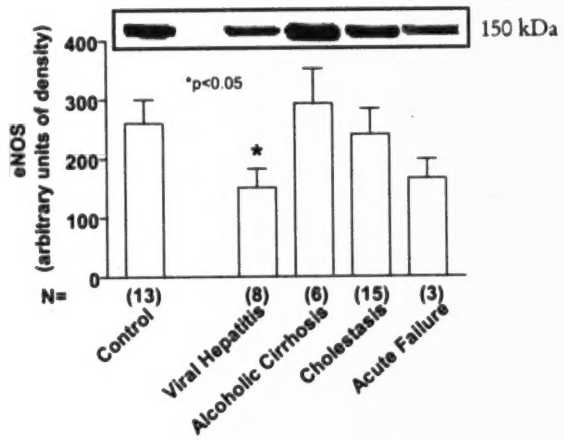


Figure II-6: **eNOS EXPRESSION IN NORMAL VS. MAJOR LIVER DISORDERS** The mean intrahepatic expression of eNOS in normal control liver is shown along with livers of various end-stage diseases. Expression is indicated in arbitrary units of density. The insert shows representative blots at the corresponding molecular weight. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

IMMUNOHISTOCHEMISTRY OF eNOS IN NORMAL LIVER VS. CHRONIC LIVER DISORDERS

The low-power micrographs seen in Figure II-7 show the results of the immunohistochemical analysis of eNOS in control and chronic diseased liver sections. In all tissue sections, control and diseased, the distribution of eNOS appeared as a rather uniform pattern lacking any discernable signs of zonation. At low power magnification, the diffuse distribution of eNOS in hepatocytes is readily seen. The high-power micrographs (Fig. II-8) revealed a greater diversity of cell types in which eNOS protein was distributed. Again, in all liver sections, control and diseased, eNOS immunoreactivity was consistently noted in the endothelium of hepatic arteries, central veins and sinusoids. Upon closer examination there is a rather uniform, strong presence of eNOS-related immunoreactivity in nuclear regions of hepatocytes. In addition to those cells already mentioned, eNOS immunoreactivity was positive in cells of the biliary epithelium.

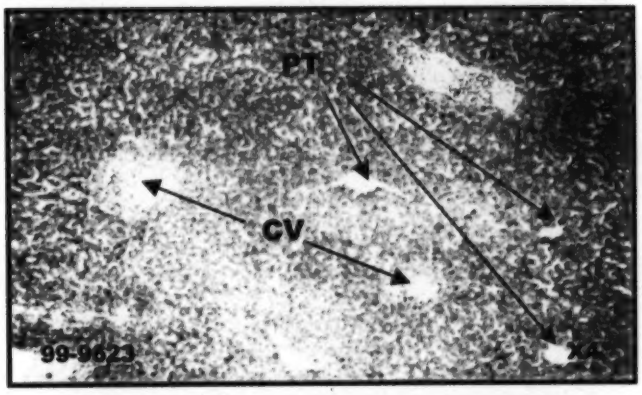
eNOS IMMUNOHISTOCHEMISTRY IN NORMAL LIVER VS. CHRONIC LIVER DISORDERS

Figure II-7A: A section of control liver is shown at 4X magnification. The portal triads (PT) and central veins (CV) are labeled. Positive brown staining for eNOS is apparent throughout the tissue section without any signs of heterogeneous distribution.

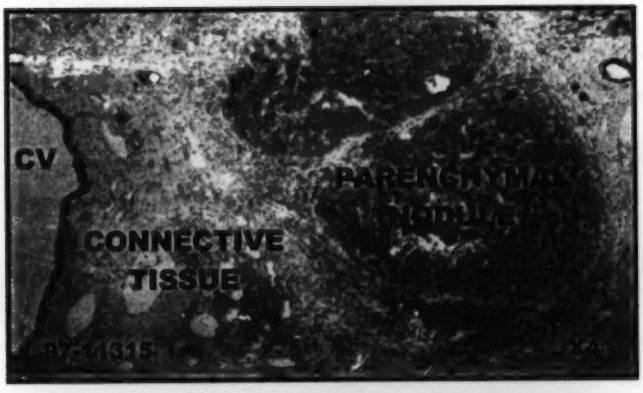
Figure II-7B: A section of liver from a patient diagnosed with alcoholic cirrhosis is shown at 4X magnification. The advanced stage of the disease leads to the development of diffuse bands of connective tissue surrounding nodules of parenchymal cells. Brown staining in the nodules appears relatively consistent at this magnification.

Figure II-7C: A section of liver from a patient diagnosed with viral hepatitis is shown at 4X magnification. Again, the advanced stage of the disease leads to the development of diffuse bands of connective tissue surrounding nodules of parenchymal cells. Brown staining in the nodules appears relatively consistent at this magnification. Bile deposits are also identifiable due to the off color staining.

A. Control



B. Alcoholic Cirrhosis



C. Viral Hepatitis

Figure II-7

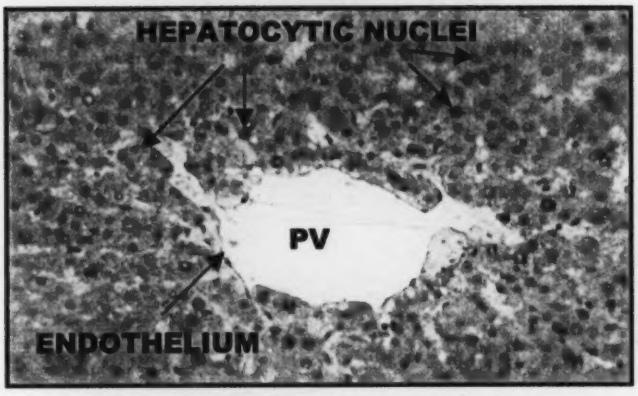
eNOS IMMUNOHISTOCHEMISTRY IN NORMAL LIVER VS. CHRONIC LIVER DISORDERS (HIGH POWER)

Figure II-8A: A section of control liver is shown at 16X magnification. The portal vein (PV) is labeled. Positive brown staining for eNOS is apparent throughout the tissue section however regions that show particularly intense staining include the endothelial lining in the PV and the hepatocytic nuclei.

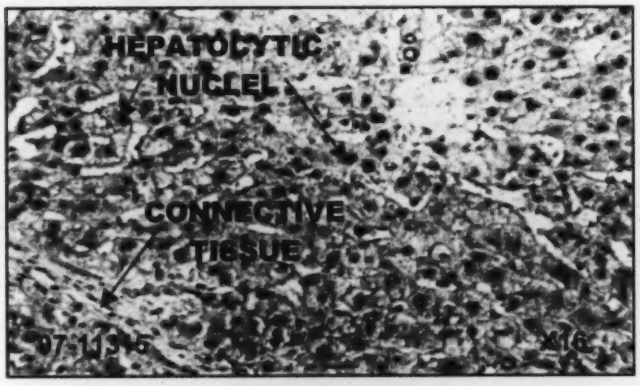
Figure II-8B: A section of liver from a patient diagnosed with alcoholic cirrhosis is shown at 16X magnification. The advanced stage of the disease leads to the development of diffuse bands of connective tissue as seen here. Brown staining in the nodules appears to be concentrated at the hepatocytic nuclei.

Figure II-8C: A section of liver from a patient diagnosed with viral hepatitis is shown at 16X magnification. Again, the advanced stage of the disease leads to the development of diffuse bands of connective tissue as seen here. Brown staining in the nodules appears to be concentrated at and around the hepatocytic nuclei.

A. Control



B. Alcoholic Cirrhosis



C. Viral Hepatitis

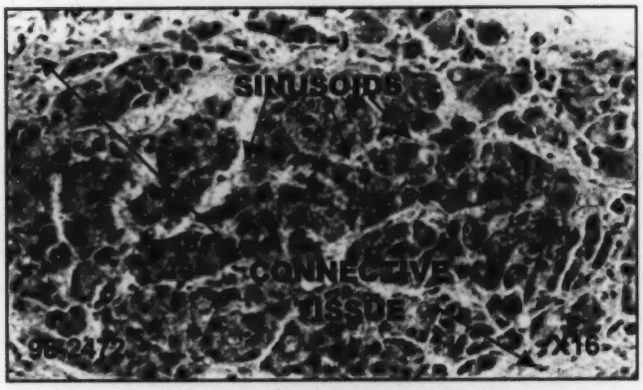


Figure II-8

iNOS ACTIVITY IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

The activity of Ca^{2+}-independent NOS in sections of control liver was 0.44 ± 0.16 pmol/min/mg protein, n = 10 (Fig. II-9). There was a significant increase in this activity in all major liver disorders except for fulminant hepatic failure.

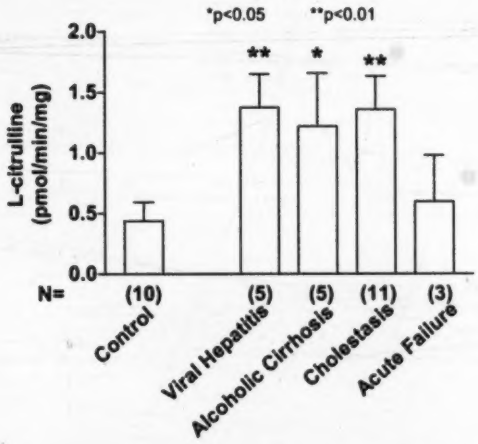


Figure II-9: **iNOS ACTIVITY IN NORMAL VS. MAJOR LIVER DISORDERS** The mean intrahepatic activity of iNOS in normal control liver is shown along with livers of various end-stage diseases. Activity is expressed as pmol L-citrulline production per minute per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

iNOS EXPRESSION IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

Western blot did not reveal significant changes in the expression of iNOS immunoreactivity in any of the patient groups when compared to control (Fig. II-10). Insert shows representative blots for each tested group of subjects.

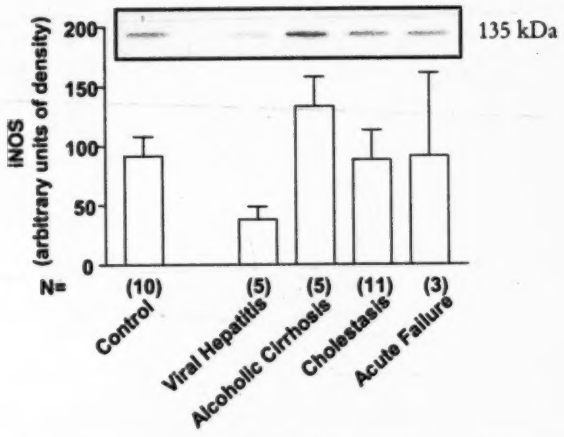


Figure II-10: **iNOS EXPRESSION IN NORMAL VS. MAJOR LIVER DISORDERS** The mean intrahepatic expression of iNOS in normal control liver is shown along with livers of various end-stage diseases. Expression is indicated in arbitrary units of density. The insert shows representative blots at the corresponding molecular weight. Number of subjects (N) is listed below. Error bars represent the SEM.

IMMUNOHISTOCHEMISTRY OF iNOS IN NORMAL LIVER VS. CHRONIC LIVER DISORDERS

The micrographs seen in Figure II-11 are representative of liver sections analyzed for the immunohistochemistry of iNOS. In normal livers, a pattern of constitutive and heterogeneous iNOS expression in the hepatic acinus was seen that appears to be symmetrically distributed around the portal triads (zone 1). In these same sections, the immunoreactivity of iNOS is considerably weaker in perivenous regions of the hepatic acinus (zone 3). In the micrographs of advanced liver fibrosis, nodules of liver cells can be seen surrounded by strands of connective tissue. Here the heterogeneous distribution of iNOS seen in less fibrotic livers and control sections is no longer apparent and parts of liver parenchyma that were spared from cirrhotic reactions display a uniform pattern of iNOS distribution among the surviving hepatocytes. A similar homogeneous pattern of iNOS immunoreactivity was also present in the sections of other chronic liver disorders such as primary biliary cirrhosis.

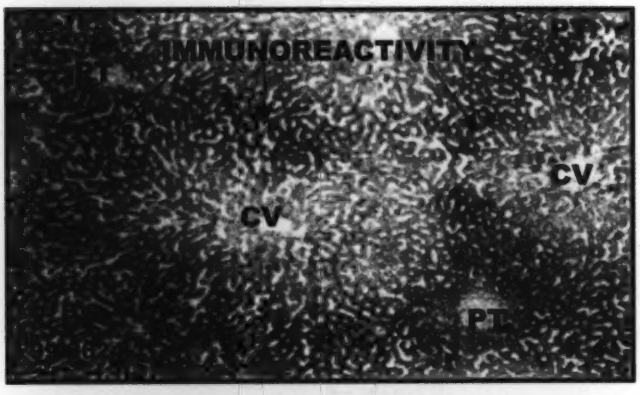
iNOS IMMUNOHISTOCHEMISTRY IN NORMAL LIVER VS. CHRONIC LIVER DISORDERS

Figure II-11A: A section of control liver is shown at 4X magnification. The portal triads (PT) and central veins (CV) are labeled. Positive brown staining for iNOS is symmetrically associated with zone 1 regions, i.e. around the PT.

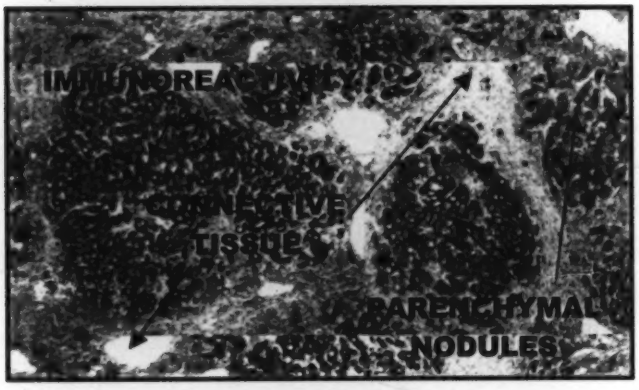
Figure II-11B: A section of liver from a patient diagnosed with α_1-Antitrypsin Deficiency Syndrome is shown at 4X magnification. The advanced stage of the disease leads to the development of diffuse bands of connective tissue as seen here. Immunoreactivity for iNOS in the nodules appears relatively uniform at this magnification..

Figure II-11C: A section of liver from a patient diagnosed with viral hepatitis is shown at 4X magnification. Again, the advanced stage of the disease leads to the development of diffuse bands of connective tissue as seen here. Brown staining in the nodules appears to be uniformly distributed.

A. Control



B. α_1-Antitrypsin Deficiency



C. Viral Hepatitis

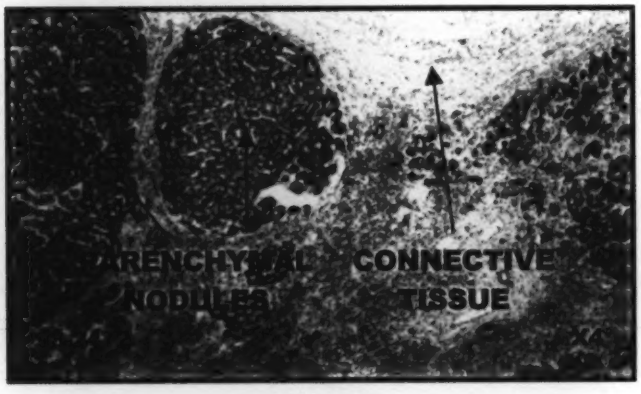


Figure II-11

IMMUNOHISTOCHEMISTRY OF iNOS IN NORMAL LIVER VS. ACUTE LIVER FAILURE

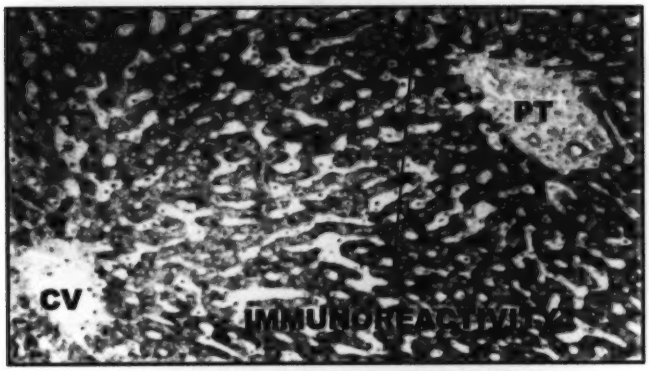
The low-power micrographs seen in Figure II-12 highlight the difference in iNOS immunoreactivity in control sections compared to livers diagnosed with acute fulminant hepatic failure. Clear evidence indicates that the immunoreactivity to iNOS was considerably lower than that seen in controls. As for the trace amounts of iNOS immunoreactivity that are present, it appears as though they are associated with the modicum of viable hepatocytes populating this tissue section.

iNOS IMMUNOHISTOCHEMISTRY IN NORMAL LIVER VS. ACUTE LIVER FAILURE

Figure II-12A: A section of control liver is shown at 10X magnification. The portal triads (PT) and central veins (CV) are labeled. Positive brown staining for iNOS is symmetrically associated with zone 1 regions, i.e. around the PT. Furthermore, staining appears to be relatively diffuse within each cell.

Figure II-12B: A section of liver from a patient undergoing liver transplant for acute liver failure is shown at 10X magnification. The fulminant nature of the disease results in widespread cell necrosis. Immunoreactivity for iNOS is sparsely evident.

A. Control



B. Acute Liver Failure

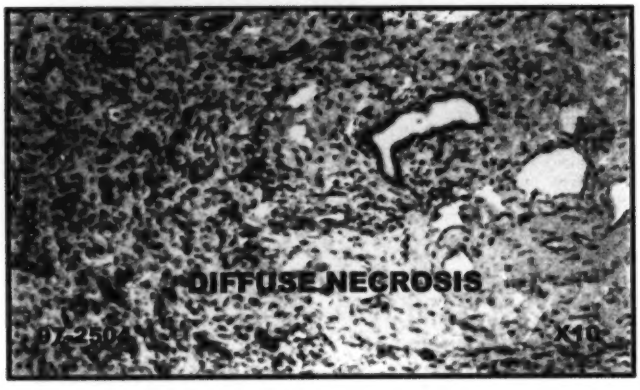


Figure II-12

ARGINASE AND NOS IN NORMAL LIVER VS. RARE LIVER DISORDERS

The activities of NOS and arginase, as well as eNOS and iNOS immunoreactivities in rare liver diseases are summarized below in Table II-4. The results shown here are statistically inconclusive due to the insufficient number of patients diagnosed with a distinct liver disease.

TABLE II-4

ARGINASE AND NOS IN NORMAL LIVER
VS. RARE LIVER DISORDERS

LIVER DISEASE (N)	ARGINASE ACTIVITY	eNOS ACTIVITY	eNOS EXPRESSION	iNOS ACTIVITY	iNOS EXPRESSION
Normal Liver (14)	24.3 ± 1.7	0.4 ± 0.1	259 ± 37.5	0.4 ± 0.2	91.0 ± 15.8
Haemochromatosis (2)	40.6	1.0	86	0.9	55
Epithelioid Hemangioendothelioma (1)	30.6	nd	210	1.3	489
Cryptogenic Cirrhosis (1)	20.6	nd	75	1.2	221
Auto-Immune Hepatitis (1)	20.6	0.1	266	1.4	47
α_1-Anti-Trypsin Deficiency (1)	20.3	nd	123	1.6	55
Budd Chiari Syndrome (1)	n/a	n/a	n/a	n/a	n/a

NOTE: The numbers of cases (N) for each form of liver disease appear in brackets. Enzyme activity and expression is expressed as pmol/min/mg protein and in arbitrary units, respectively. Computations for the normal liver group are listed as mean ± SEM. nd = not detected, n/a = data not available

IMMUNOHISTOCHEMISTRY OF iNOS IN VARIABLY CIRRHOTIC REGIONS OF BILIARY ATRESIA

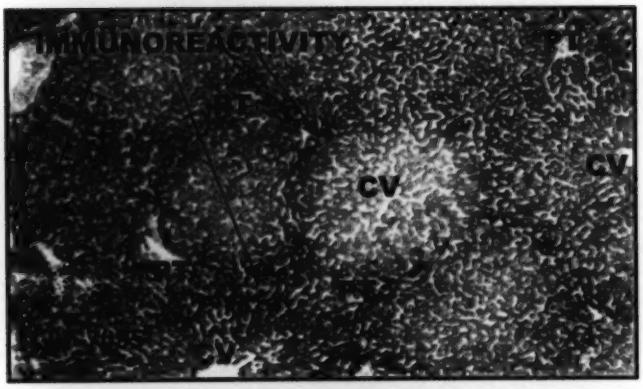
The striking difference in iNOS distribution as seen in cirrhotic livers relative to normal control sections was even observed within a single liver. In Figure II-13 the low-power micrographs of samples diagnosed with biliary atresia illustrate how iNOS in less fibrotic regions has retained the heterogeneous pattern of distribution that is apparent in controls.

iNOS IMMUNOHISTOCHEMISTRY IN NON-CIRRHOTIC AND CIRRHOTIC REGIONS OF BILIARY ATRESIA

Figure II-13A: A section of liver from a patient diagnosed with biliary atresia is shown at 4X magnification. The portal triads (PT) and central veins (CV) are labeled. Immunoreactivity for iNOS (brown staining) is symmetrically associated with zone 1 regions, i.e. around the PT.

Figure II-13B: A different region of the same liver section shown in Fig. II-13A is shown at 4X magnification. Diffuse bands of connective tissue surround parenchymal nodules within which iNOS staining is evident and of relatively consistent distribution.

A. Biliary Atresia (Non-cirrhotic)



B. Biliary Atresia (Cirrhotic)

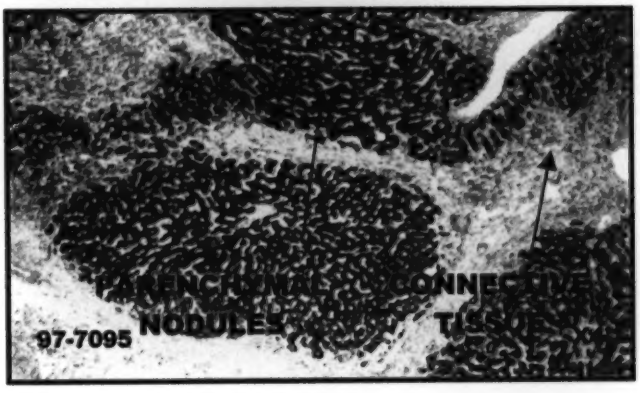


Figure II-13

PLASMA NITRITE AND NITRATE IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS

Plasma nitrite and nitrate levels vary with an individual's corresponding rate of renal clearance. To account for inter-patient variations in renal function, the ratio of plasma NO_x^- to plasma creatinine (normalized plasma NO_x^-) may be used as a standardized index of plasma NO_x^- levels.

The average normalized plasma NO_x^- level in control subjects was 0.83 ± 0.07 μM NO_x^-/μM creatinine. Prior to liver transplant there were no significant changes in plasma of patients with viral hepatitis, alcoholic cirrhosis or cholestasis. In contrast, a significant reduction of NO_x^- levels was detected in patients with acute liver failure (Fig. II-14).

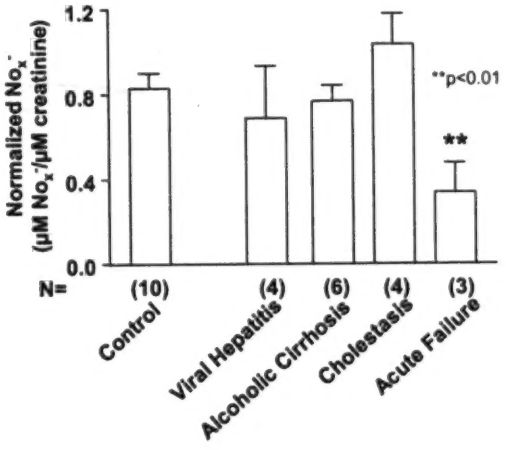


Figure II-14: **PLASMA NO_X IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS** The mean level of normalized plasma NO_x^- (ratio of NO_x^-: creatinine) is shown for healthy donors as well as for patients diagnosed with various end-stage liver diseases. Patient blood samples were collected immediately prior to liver transplantation. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

The levels of plasma NO_x^- detected in patients prior to liver transplant for rare liver disorders are shown in Table II-5. The results shown here are statistically inconclusive due to the insufficient number of patients diagnosed with a distinct liver disease.

TABLE II-5

NORMALIZED PLASMA NO_X^- IN HEALTHY DONORS VS. PATIENTS WITH RARE LIVER DISORDERS

DIAGNOSIS (N)	NORMALIZED PLASMA NO_X^-
Healthy Donor (10)	0.83 ± 0.07
Haemochromatosis (2)	0.73
Epithelioid Hemangioendothelioma (1)	1.17
Cryptogenic Cirrhosis (1)	0.46
Auto-Immune Hepatitis (1)	1.20
α_1-Anti-Trypsin Deficiency (1)	0.88
Budd Chiari Syndrome (1)	0.33

NOTE: The numbers of cases (N) for each form of liver disease appear in brackets. Normalized plasma NO_x^- is expressed as μM NO_x^-: μM urea. Computations for the healthy donor group are listed as mean ± SEM. Patient blood samples were collected immediately prior to liver transplantation.

To determine whether liver transplant has an impact on plasma NO_x^- levels they were compared for each major liver disorder at three distinct time points: Pre-op (immediately prior to liver transplantation); Post-op Day 1 (first day following surgery); and Post-op Day 5 (fifth day after surgery). To evaluate the reliability of normalized plasma NO_x^- levels the respective simple NO_x^- levels are also shown for comparison. The levels of both normalized and simple plasma NO_x^- did not differ from the control in patients diagnosed with viral hepatitis, alcoholic cirrhosis, or cholestatic disease (Figures II-15, II-16, II-17, respectively). For the patients diagnosed with fulminant hepatic failure the normalized level of plasma NO_x^- was significantly reduced on Post-op Day 5 relative to the control in contrast to there being no significant differences associated with corresponding crude plasma NO_x^- levels (Fig. II-18).

PLASMA NO_x^- AND NORMALIZED PLASMA NO_x^- IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS

Figure II-15 A-B: The mean level of plasma NO_x^- (μM) is shown in A for healthy donors (N = 10) as well as for patients (N = 3) diagnosed with viral hepatitis. Graph B shows the mean normalized levels of plasma NO_x^- (ratio of NO_x^-: creatinine) for the same patients. Error bars represent the SEM.

Figure II-16 A-B: The mean level of plasma NO_x^- (μM) is shown in A for healthy donors (N = 10) as well as for patients (N = 4) diagnosed with alcoholic cirrhosis. Graph B shows the mean normalized levels of plasma NO_x^- (ratio of NO_x^-: creatinine) for the same patients. Error bars represent the SEM.

Figure II-17 A-B: The mean level of plasma NO_x^- (μM) is shown in A for healthy donors (N = 10) as well as for patients (N = 3) diagnosed with cholestatic disease. Graph B shows the mean normalized levels of plasma NO_x^- (ratio of NO_x^-: creatinine) for the same patients. Error bars represent the SEM.

Figure II-18 A-B: The mean level of plasma NO_x^- (μM) is shown in A for healthy donors (N = 10) as well as for patients (N = 3) diagnosed with acute liver failure. Graph B shows the mean normalized levels of plasma NO_x^- (ratio of NO_x^-: creatinine) for the same patients. Error bars represent the SEM. Statistical significance is indicated by asterisk(s).

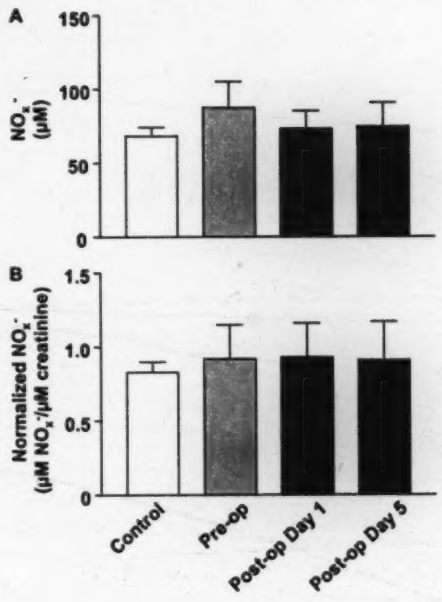


Figure II-15

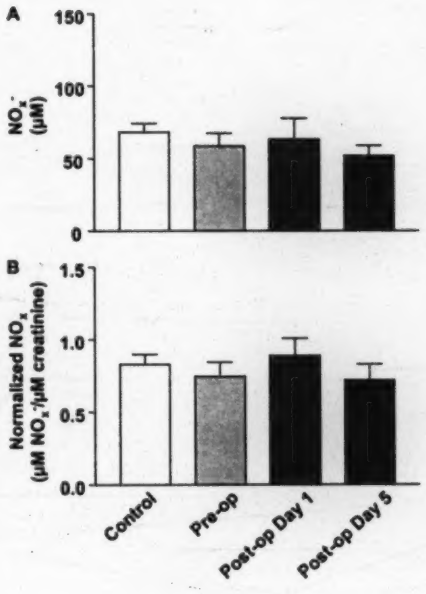


Figure II-16

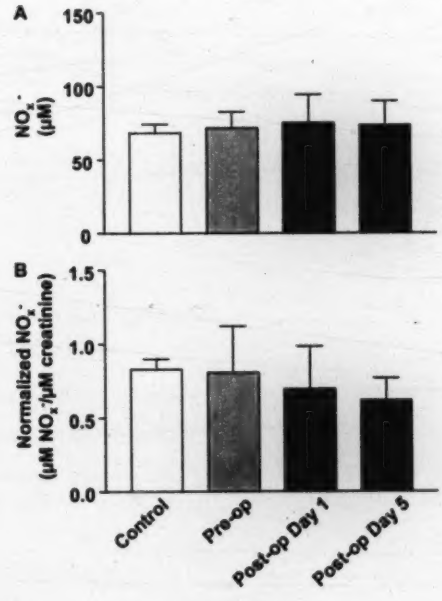


Figure II-17

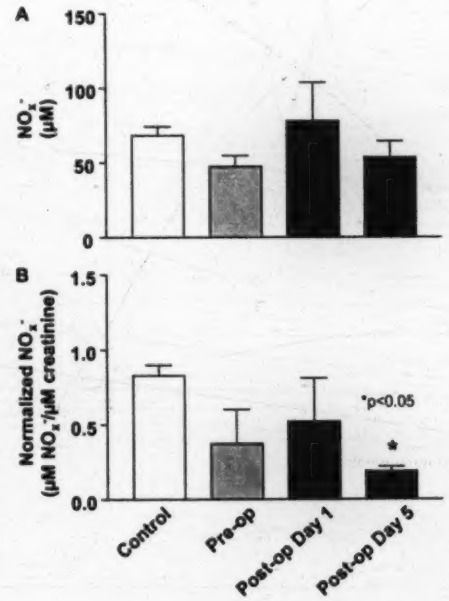


Figure II-18

MMP-2 ACTIVITY IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

The activity levels of the 72 kDa matrix metalloproteinase (proMMP-2) and the 64 kDa matrix metalloproteinase (MMP-2) were determined in liver specimens obtained from liver transplant patients and compared with controls. Figure II-19 shows the average level of proMMP-2 activity (arbitrary units of density per mg protein) determined for 13 control livers compared to several major forms of liver disease. Results show that activity was significantly elevated in all liver diseases except for fulminant hepatic failure ($n=3$; $p>0.05$). While absent in all controls, the 64 kDa isoform of MMP-2 was detected in only 3 of the 31 analyzed samples: 2 from the cholestasis group and 1 from the fulminant hepatic failure group (data not shown).

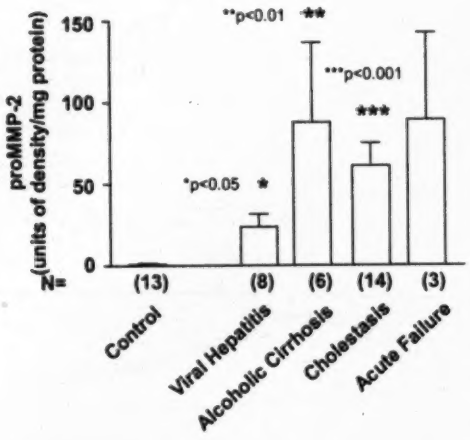


Figure II-19: **proMMP-2 ACTIVITY IN NORMAL VS. MAJOR LIVER DISORDERS**
The mean intrahepatic activity of proMMP-2 in normal control liver is shown along with livers of various end-stage diseases. Activity is expressed in terms of arbitrary units of density per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

MMP-9 ACTIVITY IN NORMAL LIVER VS. MAJOR LIVER DISORDERS

The activity levels of the 92 kDa matrix metalloproteinase (proMMP-9) and the 82 kDa matrix metalloproteinase (MMP-9) were determined in liver specimens obtained from liver transplant patients and compared with controls. Figure II-20 shows the average level of proMMP-9 activity determined for thirteen control livers compared to several major forms of liver disease. Results show that the activity in viral hepatitis, alcoholic cirrhosis and cholestasis was not significantly changed when compared to the control group. In contrast, the levels of proMMP-9 were significantly elevated in patients with acute liver failure. MMP-9 was not detected in any of the samples of human liver.

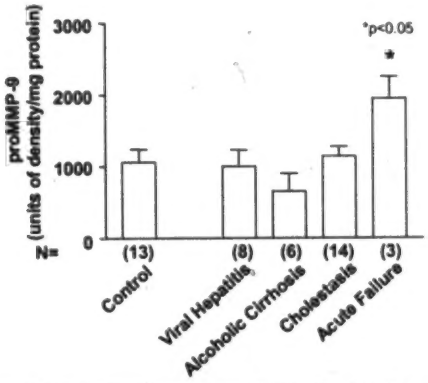


Figure II-20: **proMMP-9 ACTIVITY IN NORMAL VS. MAJOR LIVER DISORDERS**
The mean intrahepatic activity of proMMP-9 in normal control liver is shown along with livers of various end-stage diseases. Activity is expressed in terms of arbitrary units of density per mg protein. Number of subjects (N) is listed below. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

PLASMA MMP-2 ACTIVITY IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS

The activities of proMMP-2 and MMP-2 in plasma were determined in ten liver transplant patients during a perioperative time period beginning within 24 hours prior to the transplant procedure. Figure II-21 shows the average level of proMMP-2 activity (arbitrary units of density per mg protein) in blood from six healthy donors compared to liver transplant patients at 3 time points: Pre-op (immediately prior to liver transplantation); Post-op Day 1 (first day following surgery); and Post-op Day 5 (fifth day after surgery). Results show that the levels of proMMP-2 in patients were elevated prior to surgery, but normalized on the first day after surgery. MMP-2 was not detected in any of the analyzed samples.

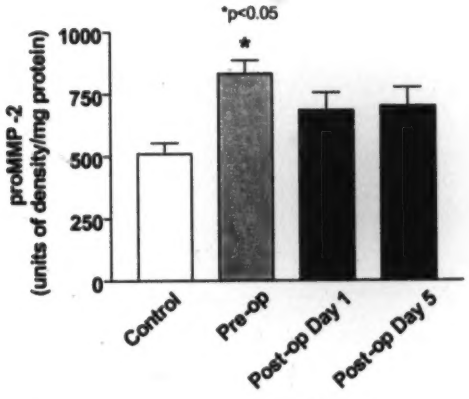


Figure II-21: **PLASMA proMMP-2 ACTIVITY IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS** The mean level of plasma proMMP-2 activity is shown for healthy donors (N=6) as well as for patients (N=10) diagnosed with various end-stage liver diseases. Activity is expressed in terms of arbitrary units of density per mg protein. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

PLASMA MMP-9 ACTIVITY IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS

The activities of proMMP-9 and MMP-9 were determined in plasma from ten liver transplant patients over a perioperative time period as defined above. Figure II-22 shows that proMMP-9 levels were significantly elevated in transplant patients as compared to controls. The levels of proMMP-9 normalized five days after surgery.

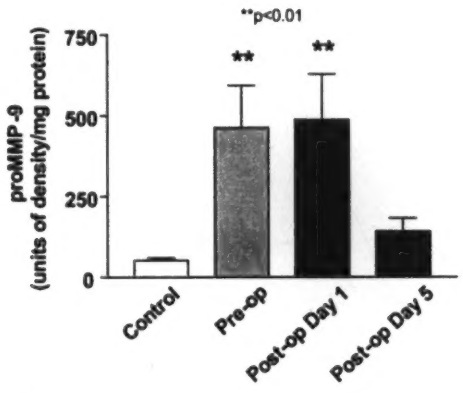


Figure II-22: **PLASMA proMMP-9 ACTIVITY IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS** The mean level of plasma proMMP-9 activity is shown for healthy donors (N=6) as well as for patients (N=10) diagnosed with various end-stage liver diseases. Activity is expressed in terms of arbitrary units of density per mg protein. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

The time-course of changes in plasma MMP-9 levels in liver transplant patients is shown in Figure II-23. No significant changes in MMP-9 levels were detected over the period of observation when compared to the control.

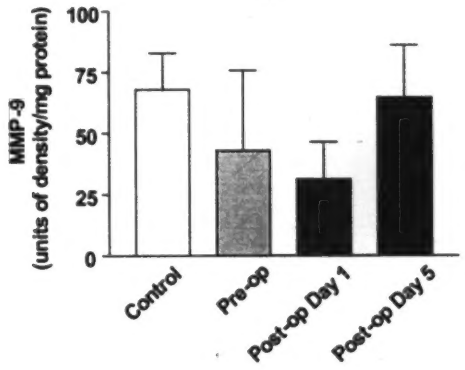


Figure II-23: **PLASMA MMP-9 ACTIVITY IN HEALTHY DONORS VS. LIVER TRANSPLANT PATIENTS** The mean level of plasma MMP-9 activity is shown for healthy donors (N = 6) as well as for patients (N = 10) diagnosed with various end-stage liver diseases. Activity is expressed in terms of arbitrary units of density per mg protein.

DISCUSSION

ARGINASE AND NITRIC OXIDE SYNTHASE IN NORMAL AND DISEASED LIVER

In healthy liver, metabolism of L-arginine via arginase and NOS is tightly regulated due to a crucial role of arginase in ammonia detoxification and NO-mediated modulation of vascular tone, haemostasis, and inflammation. In light of their common substrate dependency, we have examined the activity of these enzymes in the setting of end-stage liver disease in humans. Since arginase and NOS share the same substrate, L-arginine, it was suggested that over-expression of arginase may affect the activity of NOS[200,202] or that enhanced generation of NO could affect arginase activity.[203] This study also addresses this issue.

With an enzyme activity assay we measured the activities of hepatic arginase and NOS in supernatant collected from centrifuged liver homogenates. The results of these assays demonstrated that chronic end-stage liver diseases do not significantly affect the activity of arginase. Therefore, the subsequent experiments focused on the activity, expression, and localization of NOS in blood and diseased livers. The following sections describe the expression and possible roles of arginase and NOS in normal and diseased liver.

As expected,[202] the activity of arginase in normal human liver was high compared to NOS, accounting for over 90% of L-arginine turnover in liver homogenates. However contrary to expectations, no evidence for a significant change in arginase activity was found in various end-stage chronic liver disorders. Even when manipulating the experimental conditions, i.e. inhibiting the activity of arginase or NOS, we failed to see any change in the activity of the

uninhibited enzyme. Overall, the activities of these two enzymes in liver homogenates remained constant. Early studies using spectrophotometric methods to measure arginase activity documented a reduction in arginase activity in cirrhotic livers.[196,197,198] Curiously, these studies all used extremely high levels of L-arginine, i.e. concentrations in the mM range were used in contrast to the much more physiologic μM range we used. In this study we used a sensitive radiochemical method for the measurement of the major product of arginase, urea. The selectivity of this method was further enhanced using N^G-hydroxy-L-arginine, a selective inhibitor of this enzyme. Thus, the difference in the methodological approach used to measure enzyme activity, as well as advancements in the medical care of patients with liver failure could account for a better preservation of arginase activity in the liver of patients with chronic end-stage liver disorders that were observed in this study.

Interestingly, a catastrophic collapse of liver function brought about by acute liver failure translated into reduced activity of arginase. Massive hepatocytic necrosis (Fig. II-12) is likely to account for this observation.

We found that in normal liver the flux of L-arginine via the NOS pathway accounts for approximately 4% of total arginase turnover. The citrulline assay showed that both Ca^{2+}-dependent and Ca^{2+}-independent NOS activities were present in healthy liver. Western blot experiments determined that Ca^{2+}-dependent NOS activity could be assigned to eNOS, while iNOS accounted for the Ca^{2+}-independent NOS activity. Under the conditions used for these experiments, nNOS was not detectable.

We then went on to examine the cellular source and distribution of eNOS and iNOS in normal liver. We used immunohistochemistry of paraffin-embedded liver slices that was

combined with the antigen-retrieval technique. This approach is superior to standard immunohistochemical analysis[237] that routinely uses frozen tissue sections for NOS immunohistochemistry since the integrity of cellular fragments is better preserved in paraffin blocks.

Low-power micrographs of normal liver sections that were stained for eNOS revealed that this isoform was uniformly distributed in hepatocytes. No zonal distribution of eNOS was found. Under high-power magnification, sections of normal liver showed that eNOS immunoreactivity is also present in the endothelium of hepatic arteries, central veins and sinusoids. Interestingly, the epithelium of biliary ducts also showed strong expression of eNOS immunoreactivity. Thus, in addition to endothelial cells, both hepatocytes and biliary epithelial cells express eNOS. In the vascular compartment, the functional significance of eNOS expression and localization to the endothelial lining has been already proposed in a number of papers.[238,239] In hepatocytes however, our localization of eNOS counters a recent claim asserting that constitutive NOS is not expressed in hepatocytes.[240] Furthermore, our study provides first evidence for the presence of eNOS in human liver epithelial cells. The significance of eNOS expression in hepatocytes remains to be investigated, however, it is tempting to speculate that NO generation in hepatocytes may be involved in the regulation of metabolic functions of these cells.

There are few studies examining the role of NO in biliary epithelial cell function.[241,242] It has been suggested that NO may regulate bile canalicular motility in rat hepatocyte doublets.[243] However, our study is the first one to show the expression of eNOS in human biliary ducts where it may regulate canalicular motility in liver.

Low-power micrographs of sections of normal liver that were stained for iNOS revealed that this isoform was constitutively expressed in a heterogeneous way in the liver. The distribution of iNOS immunohistochemistry appears to be zonal with the strongest expression associated with the periportal regions and the weaker with perivenous regions of the hepatic acinus. High-power micrographs further document a dramatic cut-off between the areas of strong and weak iNOS immunohistochemistry. The constitutive expression of iNOS protein in healthy human cells and tissue is a relatively new concept in NO biology. Early investigations using cell culture systems or isolated animal organs did not detect the constitutive expression of functional iNOS protein under normal conditions.[244] However, the immunohistochemistry of normal human airway indicates that iNOS may be expressed under physiological conditions.[245] This may be due to the exposure of airways to inhaled pathogens, as the expression of iNOS activity is low in newborn rats and increases with age.[233] The data of this study obtained using Western blot and immunohistochemistry clearly indicate that there is a constitutive level of iNOS expression present in human hepatocytes.

The zonal distribution iNOS in the hepatic acinus is a subject of considerable interest. The iNOS appears to be another enzyme that is heterogeneously distributed in liver. As discussed earlier, the reasons behind the heterogenic distribution of some enzymes in the liver are not clear. In the case of iNOS, the expression of this enzyme is known to be cytokine-regulated and endotoxin plays a major role in its induction. Indeed, cytokine stimulation leads to expression of iNOS in cultured human hepatocytes.[191] I would like to hypothesize that hepatic iNOS is induced by bacterial and/or chemical products that are absorbed from the intestine to the portal circulation and then distributed to the liver. It is possible that periportal hepatocytes in the acinus encounter the greatest concentration of noxious substance when compared with

the perivenous regions. This results in a zonal induction of iNOS in the liver. The physiological significance of zonal induction of iNOS remains to be studied. First, it may be that iNOS expression under physiological conditions merely reflects pooled concentrations of intestinal toxins to which hepatocytes are exposed. Second, this expression could have a functional significance. Generation of NO by iNOS would make it readily available for local reactions of defense and non-specific immunity. Third, the induction of iNOS may represent a mechanism through which liver cells control the degree of apoptosis in the liver.[246]

The measurement of Ca^{2+}-dependent eNOS activity did not reveal any significant changes in the activity of this enzyme in patients with viral hepatitis, alcoholic cirrhosis and cholestasis. The corresponding immunoblot showed that the expression of eNOS protein was similar to control in alcoholic cirrhosis and cholestasis. There was, however, a significant reduction of eNOS immunoreactivity in patients with viral hepatitis. Interestingly, Rockey and Chung[187] evidenced that portal hypertension in rats was associated with decreased expression of eNOS mRNA and protein. Thus, reduction in eNOS may underlie hepatic injury of various origins. The immunohistochemical analysis showed that major as well as rare liver disorders examined in this study were associated with profound changes in the cellular distribution of eNOS leading to its translocation to the hepatocytic nuclei. Interestingly, growth factors such as vascular-endothelial growth factor are known to involve nuclear translocation of eNOS in vascular endothelium.[247] The significance of this observation is as yet unclear. Again, nuclear translocation of eNOS from the cell membrane may merely reflect a "growth factor storm" which is characteristic for chronic liver inflammation and cirrhosis. Alternatively, the eNOS translocation may be a part of a liver defense mechanism aimed at limiting the effects of

growth factors by decreasing the rate of cellular proliferation or apoptosis that is known to be regulated by NO.[248]

The Ca^{2+}-independent iNOS activity was significantly elevated in viral hepatitis, alcoholic cirrhosis and cholestasis. Interestingly, the degree of elevation was similar in these disease states despite their very divergent pathogenicities. Thus, induction of iNOS may be a common denominator of end-stage liver disorders of various aetiologies.

The immunohistochemistry of iNOS in chronic end-stage liver disorders showed that the parts of liver parenchyma that were spared from cirrhotic reactions strongly expressed iNOS. However, in contrast to healthy liver, the zonal distribution of iNOS immunoreactivity was lost and iNOS was uniformly present in the remaining hepatocytes. This homogeneous, strong appearance of iNOS immunoreactivity was also present in the sections of rare liver disorders such as α_1-antitrypsin deficiency syndrome.

The reasons for the loss of zonal distribution of iNOS in end-stage liver disorders are as yet unclear. However, it is likely that the presence of the cirrhotic lesion may underlie this phenomenon. The liver section obtained from a patient with biliary atresia supports this hypothesis. Biliary atresia is a paediatric disorder characterized by obstruction of extrahepatic biliary ducts. This leads to the inflammation and cirrhosis of some, but not all cell regions of the liver. Figure II-13 shows that cirrhotic sections of liver obtained from this subject show a marked loss of the zonality that is evident in non-cirrhotic regions of the same liver. It is likely that the changes in liver blood flow caused by cirrhotic processes contribute to this loss of zonality.[158]

I will further comment on the significance of these findings in the general discussion.

Measurement of NO_x^- levels in plasma is thought to be a convenient way to get an insight into the systemic release of NO. The hyperdynamic circulation state that complicates liver failure may be associated with increased release of NO.[125] In our population of patients with diverse end-stage liver disorders, we were unable to detect significant changes in plasma NO_x^- when compared with controls. Thus, plasma NO_x^- levels do not reflect well the profound alterations in NO metabolism detected in liver parenchyma of patients with viral hepatitis, alcoholic cirrhosis and cholestasis. In contrast, a reduction of NO_x^- was observed in patients with acute liver failure, again emphasizing the devastating effects of this syndrome on the generation of NO by NOS.

Interestingly, NO_x^- levels also showed no signs of change during the course of controlled surgical trauma induced by the liver transplant procedure of patients with chronic end-stage liver disorders. Again, NO_x^- levels were reduced in patients with acute liver failure who underwent the liver transplant. It is worth mentioning that the clinical protocol of patient management during the term of liver transplant includes that of an immunosuppressant, cyclosporine A (CsA). The use of this drug is associated with a reduction of NO generation[249] and these effects may confound the interpretation of NO_x^- changes in the perioperative period.

Overall, the data indicate that NO_x^- levels in plasma do not serve as a sensitive index of NO generation in chronic end-stage liver diseases, but may be a clinical marker of acute liver necrosis.

MMP-2 AND MMP-9 IN NORMAL AND DISEASED LIVER

We have examined the intrahepatic activity of MMP-2 and MMP-9 in normal liver, as well as in patients suffering from viral hepatitis, alcoholic cirrhosis, cholestasis, and acute liver failure.

Normal liver expressed low levels of proMMP-2 and proMMP-9 activity. The levels of proMMP-2 were significantly elevated in patients with viral hepatitis, alcoholic cirrhosis and cholestasis, but not in acute liver failure. These data support previous observations showing that in liver disorders associated with cirrhosis, the expression of MMP-2 may play a role in the fibrotic response of injured liver.[209] Interestingly, a different pattern of activity was found for proMMP-9. The levels of this enzyme were clearly elevated in patients with acute liver failure, but not in chronic end-stage liver disorders. It remains to be studied if a differential release of proMMP-2 vs. proMMP-9 reflects the difference between acute and chronic liver injury.

Interestingly, only 3 out of 31 samples of diseased liver showed detectable activity of MMP-2, while MMP-9 levels in all samples were below the detection limit. None of the normal liver samples showed any MMP-2 or MMP-9 activity. A review of the literature shows that the detection of *activated* isoforms of MMP-2 and MMP-9 in liver is difficult. Zymography is the only available analytical method sensitive and selective enough to differentiate between latent and activated forms of MMP-2 and MMP-9. Western blotting lacks the necessary sensitivity,[211] while antibodies used in various commercially available immunoassays do not differentiate between pro- and activated forms of these enzymes. Studies with cultured hepatic stellate cells showed that proMMP-2 and proMMP-9 are readily released by these cells, whereas only MMP-2 could be detected under these conditions.[250] Moreover, zymography of serum obtained from patients with chronic viral liver disease showed that the majority of measurable MMP-2 exists in proMMP-2 form.[212] However, it is likely that activation of proMMP-2 to MMP-2 occurs during the interactions between collagen I and hepatic myofibroblasts that are active during liver fibrosis.[209]

Some authors have shown that chronic viral liver disorders are associated with increased activity of MMP-2 in serum.[212] We have measured MMP-2 and MMP-9 in plasma rather than serum since platelets[99] and leukocytes[251] release these MMPs and this can confound the validity of obtained results.

For technical reasons we were unable to measure plasma levels of MMP-2 and MMP-9 in statistically significant numbers of patients suffering from specific liver disorders. Instead, we measured the activity of MMPs over the period of liver transplant in 10 patients (2 viral hepatitis, 3 alcoholic cirrhosis, 2 cholestasis, 1 acute liver failure and 3 different rare liver disorders). Interestingly, when analyzed as one group, the mean activities of proMMP-2 and proMMP-9 were elevated before the liver transplant. The successful transplantation resulted in a normalization of plasma MMP levels on Day 1 (proMMP-2) and Day 5 (proMMP-9) following surgery. Larger studies are needed to evaluate a possible use of plasma MMP level(s) as a clinical index of liver disease.

Chapter III

ACUTE INFLAMMATORY DISEASE

RESPIRATORY FAILURE FOLLOWING ACUTE LUNG INJURY AND INFLAMMATION

The ceaseless demand for oxygen by all living cells is no less important than the need to remove carbon dioxide that forms a potentially toxic by-product of cellular respiration. Through the cooperation of the cardiovascular and respiratory systems, these metabolic processes are met throughout one's lifetime without any conscious effort. At times however, a person may suffer an injury that results in the failure of either of these two systems. Described clinically as respiratory failure, this condition can arise from a variety of causes including acute inflammatory reactions of the lungs. Some symptoms include headache, weakness, palpitations, tachycardia, dysrhythmia, cyanosis, hypertension, oedema and coma. At this point, immediate efforts to oxygenate the patient and reverse respiratory acidosis often call for mechanical ventilation and treatment of the underlying cause(s) in an intensive care facility.

Advancements in therapy for acute inflammatory disorders are aided by continued efforts to accurately grade disease severity and characterize the underlying pathophysiologic processes. At the time of admission to an acute care facility, many patients are scored by any one of several recognized disease severity systems. Ideally, their score provides physicians with a reliable and objective means of predicting their risk of mortality and in turn selecting an effective line and intensity of therapy. In practice however these systems are each plagued by their own intrinsic limitations. As such, studies continue to focus on the identification of novel diagnostic markers that correlate with corresponding severity scores with aims at

improving the accuracy of disease severity scoring systems. The identification of these markers is likely to derive from in-depth studies on the pathologies of acute inflammatory lung injury. Data from various animal models has provided strong evidence that correlates elevated plasma levels of interleukin-6 (IL-6),[252,253] nitric oxide synthase (NOS),[252] and more recently, various matrix metalloproteinases (MMPs),[254] to acute inflammatory lung injury. In humans however, much less is known about the role of these mediators in this setting. The investigation described in this part of my thesis attempts to bridge this gap.

ACUTE PHYSIOLOGY AND CHRONIC HEALTH EVALUATION SCORE AS AN INDEX OF DISEASE SEVERITY

The *Acute Physiology and Chronic Health Evaluation* (APACHE) is a well recognized disease severity classification system that is currently in use at many of the nations leading intensive care facilities. Following improvements made to the original APACHE system, the evolution of a second generation APACHE II system has been well received.

The key parameters that are measured by the APACHE II system can be broken down into three major categories.[255] The sum of the scores from each of these constitutes the final APACHE score. The first and largest group consists of twelve acute physiologic variables that are each rated with an integer ranging from 0 to 4. As the degree of severity increases, be it abnormally high or low, the score increases as determined by a standardized, non-linear scale. The twelve variables include; temperature, mean arterial pressure, heart rate, respiratory rate, oxygenation, arterial pH, serum levels of sodium, potassium and creatinine, hematocrit, white blood count, and the Glasgow coma score. The second category determines an additional score based on the patient's age. Patients 44 years of age or less receive a zero score, whereas a higher integer is assigned for each 10 year age range, with a maximum score of six assigned to

patients 75 years or older. The third and final category applies chronic health points to previously immuno-compromised patients or those with a history of severe organ system insufficiency. A score of two is given to elective postoperative patients and five for patients who are nonoperative or emergency postoperative.

Alone however, the efficacy of the APACHE II score as a reliable risk index is limited by its design that does not take into account pathogenic factors that are involved in acute injury. Some of these agents, such as IL-6, NO and MMPs are discussed below in the context of their involvement in acute lung inflammation and injury.

INTERLEUKIN-6, NITRIC OXIDE SYNTHASE AND MATRIX METALLOPROTEINASES IN ACUTE INFLAMMATION

Interleukin-6

Although IL-6 generation is a necessary component of the immune and inflammatory response (see pg. 12: Acute Phase Proteins), an excessive and long lasting release of this cytokine appears to be associated with increased patient mortality. The dividing line between too much and too little IL-6 remains unclear. In a study of blunt trauma victims, IL-6 levels were monitored for seven consecutive days from the time of ICU admission and were correlated to each patient's injury severity score.[256] Compared to IL-6 levels in multi-trauma victims, a 1997 study found that septic shock patients have levels that correlate with outcome, are higher at all phases of therapy, and remain elevated for a longer period of time.[257] Ideally, levels of IL-6 should be measured at the earliest time in the pathologic course if they are to provide prognostic information. Altogether, despite the efforts to characterize changes in the levels of circulating IL-6 in many disease states, evidence supports that when considered alone, IL-6 provides limited prognostic information.[258,259,260]

Nitric Oxide Synthase

In the healthy lung, endogenous NO plays an important role in the regulation of airway function, modulation of immune response and continuous protection against infectious agents. Nitric oxide is generated by epithelial and endothelial cells, vascular and airway smooth muscle cells, mast cells, sensory nerves and inflammatory cells.[261] In light of their constant exposure to the environment, epithelial cells utilize eNOS to blunt the histamine contractile response of the airway tissue. Besides eNOS, nNOS is highly active and not only underlies NANC-mediated relaxation, but also serves to counteract bronchoconstriction by inhibiting cholinergic neural mechanisms.[262]

Acute inflammatory lung injury is a rapidly developing condition featuring pulmonary hypertension, arterial hypoxemia and decreased pulmonary vascular reactivity. Depending on the nature of the injurious substance and the extent of its exposure, the resulting symptoms can in some cases become syndromic.

Adult respiratory distress syndrome (ARDS) is the most severe, life threatening acute inflammatory lung injury characterized by dyspnoea, pulmonary vasoconstriction, surfactant depletion, diffuse alveolar damage and permeability oedema.[263] In particular, damage occurs in the epithelium of the alveolar-capillary barrier, giving rise to interstitial and/or alveolar oedema. Inflammatory mechanisms are maintained and regulated by a wide variety of cytokines in blood and alveolar fluid and include extensive infiltration of PMNs, activation of endothelial cells and the production of reactive oxygen species and other free radicals. In contrast to the many beneficial roles of NO in normal lung physiology, damage to the pulmonary epithelium in ARDS patients has been linked to supraphysiologic levels of NO

generated by NOS.[264,265] In accordance with these findings, it is not surprising that the level of NO in the exhaled air of patients with inflammatory lung diseases is measurably increased compared to healthy individuals, though some uncertainties still remain.[266] The validity of using such a measure as an inflammatory index has been explored in asthmatic patients, for whom elevated levels of exhaled NO are reduced following steroid therapy.[267] As might be expected, the combined presence of infiltrating leukocytes and pro-inflammatory cytokines provides just the right environment for iNOS, the implicated source of such activity. Often generated in the presence of superoxide, NO readily forms peroxynitrite, a lipid peroxidant that has also been shown to reduce lung surfactant activity *in vitro*.[268] In accordance with its lipid soluble anti-oxidant properties, NO appears to have a protective effect, in this case by preventing the conversion of surfactant to small inactive vesicles.[268] This is why, despite evidence of iNOS generation, inhaled NO appears to be of some benefit in the therapy of patients with ARDS.[269] Together with supportive therapy involving mechanical ventilation along with diuretics and/or vasorelaxants; inhalation of locally acting vasoactive concentrations of NO may benefit up to one-third of all patients.[270]

Matrix Metalloproteinases

Recent evidence has linked gelatinase activity to the development of acute lung injury following cardiopulmonary bypass in Yorkshire pigs. Results showed that lesions in alveolar-capillary basement membranes and extracellular matrix of lung tissue responsible for the clinical syndrome ARDS were completely prevented with prior i.v. administration of a chemically modified tetracycline antibiotic that selectively inhibits MMP activity.[271] In rats suffering from acute lung inflammation, extravascular albumin leakage and accumulation of

neutrophils in bronchoalveolar lavage fluids was attenuated partly with the use of anti-TIMP-2 antibodies.[272]

Using a rat model for cerebral focal ischemia, one report has shown that both MMP-2 and MMP-9 activities are significantly elevated in plasma following ischemia, and that prior administration of an antibody against MMP-9 results in a 30% reduction in infarct size.[273] Elevated plasma MMP-9 levels have also been described in patients suffering from rheumatic disease and were suggested to be an index of neutrophil activation. When values were compared between groups suffering from either acute or chronic inflammatory arthritis however, no differences were found.[274] Thus, the measure of plasma MMP activity appears to be a good index of organ inflammation and injury.

MATERIALS AND METHODS

SELECTION OF SUBJECTS

A total of 21 patients were prospectively enrolled into this study at the time of their admission to the Pulmonary Intensive Care Unit (ICU) of the University of Alberta Hospital for acute medical care requiring mechanical ventilation. Patient demographics appear at the start of the following chapter in Table III-1, and include the age and sex. The table also contains clinical data including primary diagnosis, APACHE II score, outcome and length of ICU hospitalization. Each patient provided written informed consent prior to his/her recruitment into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and those of the Research Ethics Board of the University of Alberta, Faculty of Medicine.

SPECIMEN COLLECTION

Venous blood was obtained from healthy adult donors (n = 10) who had not taken any drugs for at least 2 weeks prior to collection.

Arterial blood was drawn from each patient on two separate occasions: once at the time of ICU admission, and again on the day of his or her discharge (if applicable) from the ICU. Upon collection, 4.5 ml of blood was added to Vacutainer® tubes containing 0.5 ml of 3.8% buffered sodium citrate solution followed by rapid isolation of cell-free plasma by blood centrifugation at 800 x g for 10 min. Plasma was stored at -80 °C until assayed for IL-6, NO_x^- and MMP levels.

PROTEIN ASSAYS

Measurement of plasma protein was carried out according to the method described earlier (see pg. 80).

PLASMA IL-6 LEVELS

Measurement of plasma IL-6 levels was completed for samples from nine healthy donors and 14 patients who were admitted for intensive care. A monoclonal Quantikine™ colorimetric sandwich ELISA kit was used (R&D Systems, Minneapolis, MN). Briefly, plasma samples (100 μl) were incubated in a 96-well microtiter plate coated with monoclonal anti-human IL-6 antibodies. A second polyclonal antibody conjugated to horseradish peroxidase was added to sandwich and immobilize the bound IL-6. Unbound secondary antibodies were then washed from the plate and substrate for the enzyme was added, resulting in the generation of blue color proportional to the amound of bound IL-6. The plate was read at 450 nm in a microplate reader (BioRad Laboratories, Hercules, CA). For reference a series of 0-300 pm/ml concentrations of recombinant human IL-6 were added to the plate. The manufacturer's indicated limit of detection was ≤ 0.70 pg/ml.

PLASMA NITRITE AND NITRATE LEVELS

Measurement of plasma nitrite and nitrate levels in plasma of healthy donors and patients hospitalized for intensive care was carried out using the HPLC method described earlier (see pg. 80). One blood sample was collected at the time of each patient's admission to, and discharge from the ICU.

PLASMA MMP-2 AND MMP-9 LEVELS

The activities of gelatinase enzymes MMP-2 and MMP-9 in plasma of healthy donors and patients hospitalized for intensive care were measured by zymography using the same methods described earlier (see pg. 81).

SERUM CREATININE LEVELS

Serum levels of creatinine were obtained from patient medical records for each corresponding blood sample that was collected for use in this study. The method used to measure creatinine levels was described earlier (see pg. 83).

REAGENTS

Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM and were analyzed using the same statistical methods described earlier (see pg. 83). Here again, differences were considered statistically significant at probability values < 0.05.

RESULTS

PATIENT DIVERSITY

Table III-1 lists the pertinent demographic and clinical parameters of each patient. The mean age of the 21 patients admitted to ICU was 65.1 ± 3.9 years, consisting of 13 males and 8 females. There were 4 out of 21 patients who died before being discharged or shortly thereafter. The average length of stay on ICU was 4.1 ± 1.2 days for survivors and 24.5 ± 7.8 for non-survivors, over a range of 0.5-20 and 7-43 days, respectively. APACHE-II scores ranged from 6-38, with a mean of 18.2 ± 1.8. Sepsis accounted for the primary diagnosis in only one patient. No gender-dependent differences were detected in the measures of IL-6, NO_x^- or MMP-2 and MMP-9 in plasma from healthy donors (data not shown).

TABLE III-1

CLINICAL CHARACTERISTICS AND PERSONAL DATA OF ACUTELY ILL PATIENTS

CASE #	SEX	AGE (YRS)	PRIMARY DIAGNOSIS	APACHE II SCORE	DAYS ON ICU
1	F	80	Pulmonary Oedema	n/d	0.5
2	M	52	Respiratory Failure	6	1
3	F	22	Respiratory Failure	7	10
4	F	31	Post-op Splenectomy	8	1
5	F	85	Hip Surgery	8	1
6	M	68	Pneumonia	13	20
7	M	51	Pneumonectomy	13	1
8	F	79	Trauma	16	2
9	F	71	Pneumonia	17	8
10	M	64	Respiratory Failure	18	5
11	F	70	COPD	18	2
12	F	80	AAA	18	4
13	M	60	Respiratory Failure	21	2
14	M	75	AAA	21	4
15	M	77	COPD	21	18 †
16	M	61	Pneumonectomy	22	2
17	M	44	Sepsis	22	4
18	M	50	Necrotizing Pneumonia	22	30 †
19	M	78	Respiratory Failure	25	3
20	M	87	Ischemic Bowel Syndrome	30	7 †
21	M	83	Trauma	38	43 †

NOTE: COPD = Chronic Obstructive Pulmonary Disease, AAA = Abdominal Aortic Aneurysm, n/d = Not determined, † = Died

PLASMA IL-6 LEVELS IN HEALTHY DONORS VS. ICU PATIENTS

In control subjects, plasma IL-6 levels were below the detectable limit (< 0.70 pg/ml; n = 9). In contrast, there was a significant increase in IL-6 levels in patients ranging from 2-275 pg/ml at admission and < 0.7-135 pg/ml at discharge. The lowest level determined in the patient group at the time of admission was 2.0 pg/ml, and this level applied to the only patient treated for an abdominal-aortic aneurysm and admitted to the ICU because of respiratory failure. Figures III-1 and III-2 illustrate the variation in individual and mean IL-6 levels, respectively, of 16 patients from the time of their admission to the ICU admission to the time of their discharge.

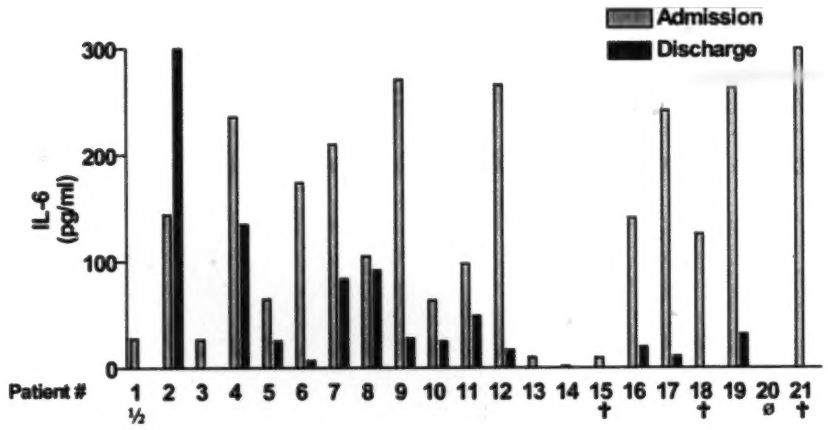


Figure III-1: **INDIVIDUALIZED PLASMA IL-6 IN PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma IL-6 levels (pg/ml) for each of the 21 patients admitted for intensive care is shown at the time of admission (grey bars) and discharge from ICU (black bars). For reference, the level of plasma IL-6 determined in 9 healthy donors was negligible (< 0.7 pg/ml), as were the discharge levels for patients 3, 13, and 14. ½ = Admission sample only, † = Admission sample only due to death, ø = Sample not available.

Figure III-2 shows the statistical averages of only the paired IL-6 levels shown in Figure III-1. Results of this grouped analysis show that the cytokine level was significiantly elevated in patients at the time of admission, followed with a significant reduction by the time of discharge. Note that the levels determined at the time of discharge were still significantly higher than the control.

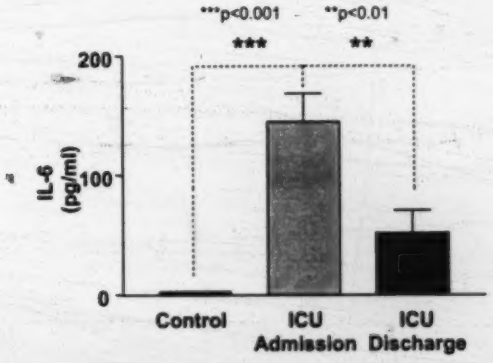


Figure III-2: **PLASMA IL-6 IN HEALTHY DONORS AND PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The mean plasma level of IL-6 (pg/ml) in healthy donors (N = 9, white bar) is shown next to the mean levels in patients (N = 16) receiving intensive care at the time of their admission (grey bar) and discharge from ICU (black bar). Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

PLASMA NO_2^- & NO_3^- LEVELS IN HEALTHY DONORS VS. ICU PATIENTS

The normal reference range of plasma NO_x^- in healthy donors was 62.5-74.3 μM (n = 10; Fig. III-3). In contrast, plasma NO_x^- levels in patients at the time of their admission and discharge from ICU ranged from 68.3-175.3 μM and 66.4-140.3 μM, respectively. Among the entire patient population (n = 21) there were no instances of subnormal levels at any time point.

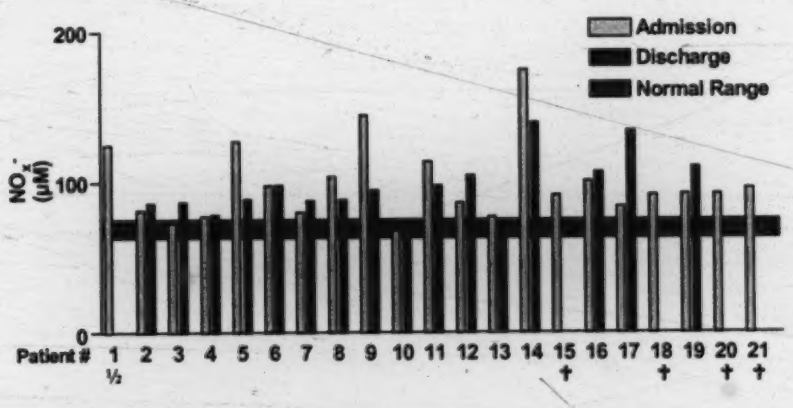


Figure III-3: **INDIVIDUALIZED PLASMA NO_x^- IN PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma NO_x^- levels (μM) for each of the 21 patients admitted for intensive care is shown at the time of admission (grey bars) and discharge from ICU (black bars). The normal range of plasma NO_x^- levels determined in 10 healthy donors is shown by the horizontal bar (62.5-74.3 μM). ½ = Admission sample only, † = Admission sample only due to death.

Figure III-4 shows the statistical averages of only the paired NO_x^- levels shown in Figure III-3. There was a significant elevation of NO_x^- levels that was detected in patients upon their admission to the ICU and these levels remained elevated upon discharge. To probe the influence of renal function on these results, we estimated and compared the elimination rates of creatinine and NO_x^- for each patient. At the time of admission we found a weak correlation between the two parameters ($r = 0.34$; $p > 0.05$; $n = 21$), however a significant correlation was found at the time of discharge ($r = 0.79$; $p < 0.001$; $n = 16$).

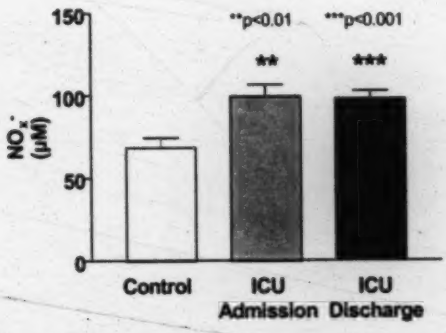


Figure III-4: **PLASMA NO_x^- IN HEALTHY DONORS AND PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma NO_x^- levels (μM) in healthy donors ($N = 10$, white bar) is shown next to the mean levels in patients ($N = 17$) receiving intensive care at the time of their admission (grey bar) and discharge from ICU (black bar). Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

PLASMA MMP-2 ACTIVITY IN HEALTHY DONORS VS. ICU PATIENTS

The plasma activity levels of proMMP-2 and MMP-2 were determined for each patient at the time of their admission to, and discharge from, ICU (Fig. III-5). These values ranged from 83-287 and 51-277 arbitrary units of density per mg protein for admission and discharge specimens, respectively. The lowest and highest admission levels pertained to patients admitted for pneumonectomy (APACHE score = 13) and sepsis (APACHE score = 22), respectively. Likewise, minimum and maximum discharge levels applied to diagnoses of respiratory failure (APACHE score = 18) and post-operative splenectomy (APACHE score = 8), respectively.

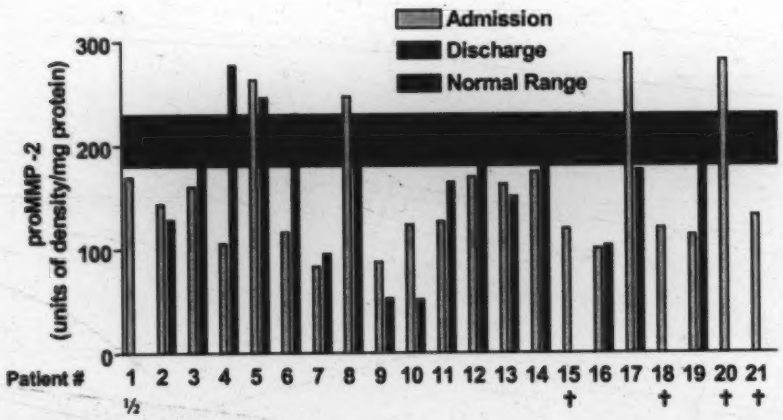


Figure III-5: **INDIVIDUALIZED PLASMA proMMP-2 LEVELS IN PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma proMMP-2 level (arbitrary units of density per mg protein) for each of the 21 patients admitted for intensive care is shown at the time of admission (grey bars) and discharge from ICU (black bars). The normal range of plasma proMMP-2 levels determined in 4 healthy donors is shown by the horizontal bar (181-230 units). ½ = Admission sample only, † = Admission sample only due to death.

Figure III-6 shows the statistical averages of only the paired proMMP-2 levels shown in Figure III-5. There was no significant difference between the levels of proMMP-2 in plasma of patients when compared to controls (Fig. III-6). The activity of MMP-2 was not detected in any plasma samples under the conditions of our experiments.

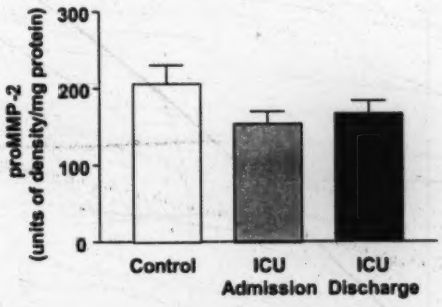


Figure III-6: **PLASMA proMMP-2 IN HEALTHY DONORS AND PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The mean level of plasma proMMP-2 activity is shown for healthy donors (N = 4, white bar) as well as for patients (N = 16) receiving intensive care at the time of their admission (grey bar) and discharge from ICU (black bar). Activity is expressed in terms of arbitrary units of density per mg protein. Error bars represent the SEM.

PLASMA MMP-9 ACTIVITY IN HEALTHY DONORS VS. ICU PATIENTS

Figure III-7 shows the individual plasma proMMP-9 levels for which the activity ranges of admission and discharge values were 20-1602 and 20-810 arbitrary units of density per mg protein, respectively. The lowest and highest admission level of IL-6 pertained to a patient admitted for motor vehicle accident trauma (highest recorded APACHE score of 38) and another for necrotizing pneumonia (APACHE score = 22), respectively. Likewise, minimum and maximum discharge levels applied to diagnoses of pneumonia (APACHE score = 13) and motor vehicle accident trauma (APACHE score = 16), respectively.

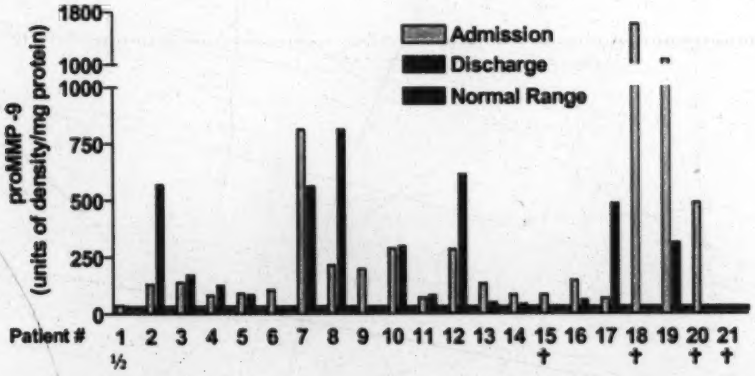


Figure III-7: **INDIVIDUALIZED PLASMA proMMP-9 LEVELS IN PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma proMMP-9 level (arbitrary units of density per mg protein) for each of the 21 patients admitted for intensive care is shown at the time of admission (grey bars) and discharge from ICU (black bars). The normal range of plasma proMMP-9 levels determined in 4 healthy donors is shown by the horizontal bar (18-30 units). ½ = Admission sample only, † = Admission sample only due to death.

Figure III-8 shows the statistical averages of only the paired proMMP-9 levels shown in Figure III-7. There was a significant increase in the plasma activity of proMMP-9, which remained high at the time of discharge.

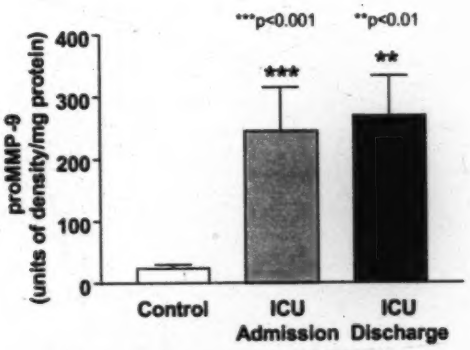


Figure III-8: **PLASMA proMMP-9 IN HEALTHY DONORS AND PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The mean level of plasma proMMP-9 activity is shown for healthy donors (N = 4, white bar) as well as for patients (N = 16) receiving intensive care at the time of their admission (grey bar) and discharge from ICU (black bar). Activity is expressed in terms of arbitrary units of density per mg protein. Error bars represent the SEM. Statistical significance is indicated above by asterisk(s).

The levels of plasma MMP-9 activity are shown below for each patient (Fig. III-9). At the time of admission and discharge from ICU they ranged from 8-74 and 0-104 arbitrary units of density per mg protein, respectively. The lowest and highest admission levels pertained to patients admitted for COPD (Apache score = 21) and trauma (Apache score = 17), respectively. Likewise, the minimum and maximum discharge levels both applied to cases of pneumonia for which the respective Apache scores were 17 and 13.

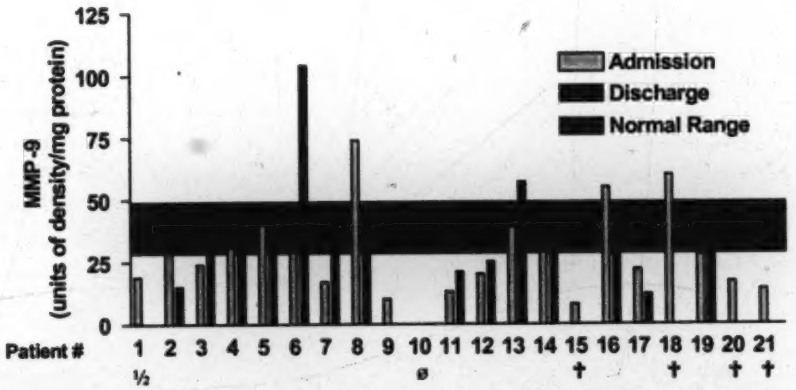


Figure III-9: **INDIVIDUALIZED PLASMA MMP-9 LEVELS IN PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The plasma MMP-9 level (arbitrary units of density per mg protein) for each of the 21 patients admitted for intensive care is shown at the time of admission (grey bars) and discharge from ICU (black bars). The normal range of plasma MMP-9 levels determined in 4 healthy donors is shown by the horizontal bar (29-49 units). ½ = Admission sample only, † = Admission sample only due to death, ø = Sample not available.

Figure III-10 shows the statistical averages of only the paired MMP-9 activity levels shown in Figure III-9.

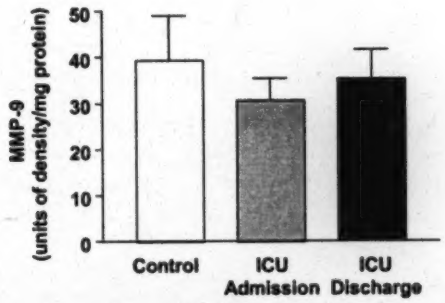


Figure III-10: **PLASMA MMP-9 IN HEALTHY DONORS AND PATIENTS DURING ADMISSION VS. DISCHARGE FROM ICU** The mean level of plasma MMP-9 activity is shown for healthy donors (N = 4, white bar) as well as for patients (N = 15) receiving intensive care at the time of their admission (grey bar) and discharge from ICU (black bar). Activity is expressed in terms of arbitrary units of density per mg protein. Error bars represent the SEM.

Addition of the MMP inhibitor 1,10-phenanthroline to the incubation medium resulted in the complete disappearance of all bands associated with the MMP-activities that are described here (data not shown).

As an added precaution we also looked into the possibility that plasma MMP levels fluctuate significantly in healthy individuals. To do so we measured the short-term variability of plasma gelatinase activity (MMP-2 and MMP-9) in healthy blood donors and found no evidence of change over a 7-day interval (data not shown).

CORRELATIONS OF PLASMA MARKERS AND THE APACHE II SCORE

Table III-2 illustrates the correlations between the APACHE II score, assigned to each patient upon admission to ICU, and the admission levels of plasma markers (described above) including IL-6, NO_x^-, normalized NO_x^-, proMMP-2, proMMP-9 and MMP-9. For all markers, no significant correlations were found.

TABLE III-2

LACK OF CORRELATION BETWEEN PLASMA MARKERS AND THE APACHE II SCORE

ADMISSION LEVEL	IL-6	NO_x^-	NO_x^-:Cre	proMMP-2	proMMP-9	MMP-9
Apache Score	r=0.27	r=0.09	r=-0.11	r=0.08	r=0.18	r=-0.02
	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
	n=19	n=20	n=20	n=20	n=20	n=20

NOTE: NO_x^-:Cre = Normalized plasma nitrite and nitrate, r = Pearson correlation coefficient, p = probability coefficient, n = Number of cases

DISCUSSION

The overall objective of this study was to analyze the expression and possible role of crucial mediators in the development of systemic acute inflammatory reactions involving the lungs. We selected a heterogeneous population of critically ill patients diagnosed with various primary respiratory disorders (e.g. pneumonia, necrotizing pneumonia and chronic obstructive pulmonary disease), as well as with diseases in which lungs were secondarily involved (e.g. sepsis and ischemic bowel syndrome). However, the respiratory failure was a common clinical and pathophysiologic denominator of this group of patients indicating that the lungs were critically involved in acute injury and inflammation.

In the setting of life-threatening human disease, plasma is the only readily accessible source of material for experimental purposes. Therefore, we have measured plasma levels of IL-6, NO, MMP-2 and MMP-9 in critically ill patients. The results show that acute inflammation in humans, similar to its profile in animal counterparts, is clearly associated with the systemic mediator release.

Interleukin-6 levels, as befitted a cytokine of early immune response, were dramatically elevated in all patients admitted to ICU. The measurements of plasma IL-6 confirmed its widespread elevation at the time of each patient's admission to the ICU for various acute inflammatory diseases requiring respiratory support. It has previously been found that prolonged and excessive elevation of IL-6 during chronic inflammation increases mortality. Elevated levels of IL-6 can be detected in as little as 90 min following skin incision,[275] though the extent and duration of such levels appears to depend on the nature and outcome of the

surgical procedure. In the current study we detected a rather short-lived burst of IL-6 lasting less than an average of 4.1 ± 1.2 days. This can best be explained by its previously reported half-life of 4 hours.[276]

In contrast to the short-lasting increase in IL-6 levels, we detected a prolonged increase in plasma NO_x^- levels that were still elevated upon discharge of patients from the ICU, i.e. when their condition was no longer critical. The logistics of our study precluded us from monitoring the levels of NO metabolites in these patients outside the ICU. There is also evidence for reciprocal interactions between IL-6 and NO. In combination with other pro-inflammatory cytokines, IL-6 has been shown to promote the induction of iNOS.[277] Interestingly, NO generation has also been shown to inhibit IL-6 generation.[64,65] A sustained elevation of plasma NO_x^- may, in part, explain the reduction in IL-6 that has been observed in our study.

Like IL-6 and NO, MMPs in plasma may very well represent an index of their global generation. Of the many isoforms known, MMP-2 and MMP-9, members of the gelatinase group, have received considerable interest due to the nature of their substrate specificity (collagen types IV, V, VII, X and elastin)[95] and distribution. Due to its constitutive nature, MMP-2 is widely expressed and mostly dependent on post-translational regulation. The results presented here show no variation in MMP-2 levels for the patient group, thus reflecting the general housekeeping role of this enzyme. In contrast to the ubiquitous distribution of MMP-2, immune cells are a primary source of MMP-9. Like NOS, MMP-9 is sensitive to pro-inflammatory cytokines like IL-1β and TNF-α, and it is through such pathways that MMP-9 is thought to facilitate the neutrophil infiltration that marks the onset of the inflammatory process.[111,112] In one study, elevated plasma levels of MMP-9 in patients with rheumatic disease

were validated as an index of neutrophil activation.[274] Interestingly, there may be also the cross-talk between MMPs, IL-1 and NO. Although it is unclear if IL-6 is involved in MMP induction[278] there is evidence that implicates NO in the regulation of MMP release.[98] Quite recently, NO or $ONOO^-$ were shown to induce and activate MMPs in vitro.[279,128,233]

In the clinical setting it is difficult to investigate the pathophysiological and clinical significance of mediator generation and release. This can only be achieved once selective inhibitors of mediator generation or action are approved for human use in inflammation. Interleukin-6 has not been pharmacologically targeted to date as an inflammatory mediator, as the issue "How much IL-6 is crucial for an effective immune response and how much may be detrimental?" has not been decided upon yet. Instead, IL-6 levels have been measured as "a guiding companion" when evaluating the antiinflammatory effects of anti-TNF antibodies.[280] Unfortunately, the anti-TNF therapy is yet to fulfill its antiinflammatory potential. Similarly, the therapeutic window of isoform-non-selective NOS inhibitors appears to be very narrow, e.g. a recent clinical trial organized by GlaxoWellcome to evaluate the efficacy of NOS inhibition in sepsis was terminated because of toxic effects of the inhibitor. Lately, inhibitors of MMPs have entered clinical trials to explore their use in the treatment of cancer,[281,282] periodontal disease[283] and rheumatoid arthritis.[284] Therefore, in our investigation, in order to study the significance of IL-6, NO and MMPs in acute inflammatory injury, we have correlated plasma levels of these mediators with available clinical parameters including the APACHE II score of disease severity. We did not find a significant correlation between the levels of investigated mediators and clinical indices of the disease, including the APACHE II score. A number of reasons could account for such results. First, the size of our group, although adequate for mediator measurements, is rather small for population statistics. Second, we have

selected a heterogeneous group of patients despite a heavy involvement of acute lung inflammation and injury in each case. Indeed, a number of recent studies conducted in acute care settings on more homogenous groups of patients have uncovered a tenacious relationship between plasma IL-6 levels and injury status. In a group of blunt trauma victims, IL-6 levels were monitored for 7 consecutive days from the time of ICU admission and were correlated to each patient's injury severity score.[256] Compared to multi-trauma victims, a 1997 study found that septic shock patients have circulating IL-6 levels that correlate with outcome, are higher at all phases of therapy, and remain elevated for a longer period of time.[257] Third, the APACHE II score and the indices of critical disease mainly address the overall physiopathological status of the patient rather than the clinical and biochemical symptoms of inflammation. Clearly, more studies are needed to evaluate the relationship between the release of mediators of inflammation and injury in the setting of human disorders.

Chapter IV

GENERAL DISCUSSION AND CONCLUSIONS

My Ph.D. studies have dealt with the generation and release of major inflammatory mediators during acute and chronic inflammation in the setting of critical human disorders. In particular I have focused on several ubiquitous mediators: arginase, NOS, IL-6 and MMPs.

For a clinical model of chronic inflammation we selected patients suffering from end-stage chronic liver diseases. The end-stage status required each patient to undergo an orthotopic liver transplant that was coordinated and carried out through the Liver Transplantation Program at the University of Alberta Hospital. While the majority of patients presented with severe cirrhosis and liver failure, the pathogenetic factors underlying each liver dysfunction were heterogeneous. In general, these included viral infection, chronic alcoholism, cholestatic diseases and genetically conditioned rare liver disorders such as α_1-antitrypsin deficiency and haemochromatosis. In the course of this study we obtained surgical sections of over 60 diseased livers for the purpose of carrying out the various mediator assays. In addition, we collected perioperative blood samples from each liver transplant patient. Finally, we investigated and resolved some crucial histologic aspects of liver disease. For comparison, the few studies of human liver disease that were conducted prior to this investigation focused only on the identification of cDNA or mRNA for NOS.

To study the activity of NOS, IL-6 and MMPs during acute inflammation, a heterogeneous population of patients presenting with acute pulmonary failure was studied. The population included patients with primary lung disorders, as well as those with disorders in which

pulmonary failure occurred secondarily, i.e. as a result of systemic complications of the underlying disease. Again, thanks to the well-coordinated support of the ICU research group, nursing staff and physicians, we obtained sufficient amounts of patient plasma to carry out the enzyme activity assays.

Nitric Oxide and Arginase

Major novel observations obtained by this study relate to the characterization of NOS enzymes in normal and diseased liver. First is that, hepatocytes are the major source of both eNOS and iNOS enzymes in human liver. This claim is based upon the immunohistochemistry results and the fact that these cells occupy over 90% of liver tissue. Second, a differential distribution of these isoforms (uniformly distributed eNOS vs. periportally distributed iNOS) suggests that these enzymes have multiple functional properties. Third, heterogeneous chronic liver disorders including viral hepatitis, alcoholic cirrhosis and cholestasis all showed uniformly homogenous, strong expression of iNOS protein in addition to correspondingly high levels of iNOS activity. Last, the immunohistochemical analysis of cirrhotic vs. non-cirrhotic regions of liver with biliary atresia indicates that the changes in iNOS that accompany a wide range of end-stage chronic liver disorders may be cirrhosis-dependent.

The selective, universal increase in iNOS activity that we detected in all investigated end-stage liver diseases is intriguing. Three scenarios dealing with the possible significance of these observations need to be addressed. First, it may be that the homogenous and strong expression of iNOS merely reflects changes in the blood flow observed in cirrhotic liver. A compelling indication to support this notion comes from the analysis of slides from a patient

with biliary atresia showing that diseased sections of liver uniformly express iNOS, while in normal regions the zonality of iNOS distribution remains intact. The remaining two scenarios have a profound pharmacological significance. Assuming that isolated and increased expression of iNOS exerts detrimental effects on liver function, this would build the case to evaluate the effects of selective inhibitors of iNOS as a measure to delay or prevent liver failure due to liver inflammation and cirrhosis. A difficulty associated with this approach is that the zonal expression of iNOS in the periportal regions may be a matter of physiologic immune significance, and as such would need to be preserved. This poses a similar puzzling question that has been troubling the developers of IL-6-based therapies: "How much of inducible NO is too much and thus represents a pathological threat?". The third scenario assumes that the induction of iNOS in the perivenous areas of diseased liver represents a defence mechanism to protect disease-free hepatocytes from ischemia and damage brought about by cirrhotic insult. In this case, the substitution therapy with NO gas or NO donors could be appropriate. A certain compromise between the second and third scenario would be to use a combined therapy with selective inhibitors of iNOS and NO donors. These important issues can be only addressed in animal experimentation. However, most of the available methods of experimental liver damage are based on acute liver injury induced with hepatotoxic agents[285] and thus may not accurately simulate the actual human pathology.

We have also investigated whether measurements of plasma NO_x^- levels may be used as an index of NOS activation, inflammation and injury in chronic end stage liver disease and acute lung dysfunction. This is an important issue since the usefulness of this assay has been a subject of great debate.[286] We have found that in patients with end-stage liver disorders, despite the changes in NOS expression in hepatocytes as described above, plasma NO_x^- levels

are not significantly altered when compared with controls. Thus, this parameter is of little value when investigating the clinical aspects of chronic inflammation involving NOS. In contrast, NO_x^- levels are significantly elevated during acute inflammation and injury exemplified by liver transplant procedure or acute lung injury. The clinical value of these observations is, however, uncertain since no significant correlations between patient status and elevated NO_x^- levels have been found. Larger studies focusing on a particular disease entity may be needed in order to address this issue.

Contrary to expectations deriving from early studies in the 1960's[196] and 1970's[197,198] we did not observe significant changes in the activity of arginase in patients with end-stage chronic liver disorders when compared with controls. Among some of the more salient differences between these earlier studies and the current investigation is the fact that only we examined livers that were in the end-stage of the disease process. Also, in this investigation we used L-NAME (an L-arginine analog and non-selective NOS inhibitor) and L-NOHA (an intermediate of NO synthesis and selective inhibitor of arginase) to verify that the radioactive products derived from L-arginine were indeed citrulline and urea, respectively. As L-NOHA is a new addition to a rather small family of pharmacological inhibitors of arginase, the availability of this important tool could account for the discrepancy between this and other studies. Moreover, a review of the methodologies used in the earlier studies reveals the use of substantially higher L-arginine concentrations, and colorimetric analysis was completed over a shorter incubation period. The colorimetric method has subsequently been shown to be less sensitive than radiometric methods like the one used here.[230]

An earlier study of the flux of L-arginine via arginase and NOS pathways using cultured

endothelial cells demonstrated that induction of NOS could lead to a significant depression in arginase activity.[203] Alternately, another group has found that upregulation of arginase in rat liver can lead to a reduction in NOS activity.[200] The results of our studies show that (1) arginase is not upregulated in diseased liver, and (2) inhibition of arginase or NOS in liver homogenates does not affect the activity of the other, i.e. these enzymes do not appear to engage in reciprocal inhibition in liver. These findings suggest that reciprocal inhibition between arginase and NOS is an extrahepatic phenomenon that does not take place in human liver under pathologically relevant conditions.

Matrix Metalloproteinases

We have found elevated levels of intrahepatic proMMP-2 in end-stage chronic liver disorders. These results are not surprising given the degree of liver cirrhosis detected in these patients. In contrast to proMMP-2, intrahepatic levels of proMMP-9 were elevated only in acute liver failure. Interestingly, increases in proMMP-2 and pro-MMP-9 levels in plasma were also found. This is in variation to plasma NO_x^- levels that were not modified by the disease process. Moreover, a successful liver transplantation resulted in a rapid normalization of systemic levels of proMMP-2 (within one day following operation) and proMMP-9 (by the fifth day following operation). Thus, the measurement of MMP levels in plasma appears to correlate with liver cirrhosis.

In our investigation of acute inflammatory diseases, we found only the plasma levels of proMMP-9, and not proMMP-2, were elevated. Even when these patients had recovered enough to be discharged from the ICU, their plasma levels of proMMP-9 were still significantly high. Compared to the rise in plasma proMMP-2 levels we detected in patients with chronic liver disease, the levels of this enzyme in acutely ill patients were normal. Hence,

it would seem that plasma levels of proMMP-2 may be associated more so with the chronic, rather than acute, disease entities.

As with over expression of iNOS, the pharmacological relevance of these results must await for the results of carefully designed animal studies involving relevant models of chronic liver disease and acute inflammatory disease. However, since a number of selective MMP inhibitors are now well into clinical trials to evaluate their effectiveness in various types of cancer and chronic inflammation, the stage is set for the potential use of these compounds to influence the course of tissue destruction encountered in these pathologies.[281,283,284]

In conclusion, this study has provided firm evidence that inflammatory mediators such as NO and MMPs are generated and released during both chronic and acute inflammatory disorders in humans. These observations are important, not only for the purpose of understanding the pathogenesis of inflammatory liver and lung disorders in humans, but also to provide further rationale for selective pharmacological development of anti-inflammatory drugs.

FUTURE DIRECTIONS

The results of this work include a number of novel findings that should impact the direction of future studies in a number of ways.

As was suggested earlier, a return to animal studies using suitable models could now provide some crucial answers to the pertinent questions that arise from this research. Our findings dealing with NOS in chronic and acute liver disease have focused on liver at the end-stage of the disease process. Animal models of acute and chronic liver disease would permit the analysis of intrahepatic NOS at various stages of disease progression, and in this way, may provide some clues to the nature of events that trigger the shift in NOS distribution that we have observed. Furthermore, the functional significance of these changes might also be explored in intact animals through the use of available selective inhibitors of NOS or NO donors. As well, investigations designed to isolate zone-specific hepatocytes would be useful in explaining the underlying mechanisms responsible for heterogeneous NOS protein expression in liver. Selective blockage of the tyrosine kinase pathway that is activated following vascular endothelial growth factor stimulation may help to elucidate its role in the nuclear translocation of eNOS.

Being a relatively new approach, the measurement of circulating MMP levels in blood largely remains an open field of investigation. The differential expression of plasma MMPs seen in chronic vs. acute diseases are intriguing. The use of reverse zymography would yield valuable information on the circulating level of TIMPs that have not been explored in this work.

Future efforts to acquire larger groups of patients suffering from a *specific* desease entity could help explain the temporal and pathological factors that affect the levels of MMPs and TIMPs.

The crucial role of cytokines in the inflammatory cascade cannot be fully interpreted from the measurement of IL-6 alone. Simultaneous measurement of circulating levels of TNF, IL-1 and IL-6 in patients diagnosed with acute and chronic inflammatory diseases would provide a more complete foundation on which to describe the relationship between these regulatory molecules and the levels of downstream mediators like NOS and MMPs.

NO_x^- levels are significantly elevated during acute inflammation and injury exemplified by acute lung injury. The clinical value of this observation is, however, uncertain since no significant correlations between patient status and elevated NO_x^- levels have been found. Here again, larger studies focusing on a particular disease entity could be used to further explore this observation.

CHAPTER V

BIBLIOGRAPHY

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